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(54) Title: HYBRID Fc RECEPTOR MOLECULES (57) Abstract A novel immunoglobulin binding molecule is provided being a hybrid of two or more binding molecules such as to provide a hybrid immunoglobulin binding molecule having chimeric properties characteristic of two or more individual immunoglobulin binding molecules in one molecule. Methods of production and applications of such hybrid molecules is also provided.		

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TITLE: Hybrid Fc Receptor Molecules

This invention relates to immunoglobulin binding molecules and in particular to Fc receptor (FcR) molecules and hybrids or chimeric forms thereof capable of binding one or a plurality of classes of antibody.

The invention also encompasses the different forms of Fc receptor molecules including soluble, unbound and bound forms as protein molecules and nucleotide sequences coding therefore, methods of production of hybrid Fc receptor molecules, uses of hybrid Fc receptor molecules including diagnostic testing and pharmaceutical application, poly and monoclonal antibodies detecting hybrid Fc receptor molecules and the uses thereof.

To date the Fc receptor molecules and cDNA clones thereof have been limited to specific antibody class targets for example IgG, IgE, etc.

Receptors for immunoglobulin (Fc receptors or FcR) play key roles in the immune response in allergy and in resistance to infection (1-3). On phagocytes they are responsible for the binding and removal of immune complexes. On T and B cells they are involved in signalling and in the regulation of antibody synthesis (1,2). Secreted Fc receptor related molecules (immunoglobulin binding factors) have been defined that also regulate antibody synthesis and membrane bound or soluble FcR play a role in T cell function (2). The role of FcR in allergy has also been elucidated and the binding of IgE to its receptor plays a pivotal role in these conditions (3).

In addition, to this wide range of function, FcR receptors for all immunoglobulin classes have been defined. In man and the mouse two classes of receptors for IgG have been defined and are designated FcγRI, FcγRII. In addition a third class of receptor FcγRIII has been identified in man. These receptors can be distinguished by (i) structural differences, (ii) affinity of binding of antibody, (iii) specificity for Ig classes or subclasses and (iv) reaction with monoclonal antibodies to FcR. Thus, FcγRI is a high affinity receptor for monomeric IgG whereas FcγRII and FcγRIII bind monomeric IgG with low affinity but bind immune complexes with high avidity (1-2).

Molecular cloning of cDNA or genes encoding these receptors has demonstrated that they are homologous proteins that have evolved to perform their

unique functions (4,5,6,7). Indeed these cloning experiments have shown that not only are there multiple forms of each of FcγRI, FcγRII in man and mouse (and FcγRIII in man) but that FcγRs are related (4-10). Furthermore, the receptors for IgG and IgE are also related at the nucleotide and protein levels (5,6,11,12).

The interaction of immunoglobulins with FcR was investigated by constructing chimeric FcγR. Mouse FcγRI and FcγRII cDNAs have been cloned and encode related but distinct proteins. FcγRI and II are both typical membrane spanning proteins (4,5,7). The extracellular region of FcγRI is organised into three disulphide bonded domains. By contrast, the extracellular region of FcγRII is composed of only two disulphide bonded domains. Domains 1 and 2 (D1 and D2) of FcγRI are homologous to domains 1 and 2 (d1 and d2) of FcγRII showing approximately 47% amino acid homology and D3 of FcγRI is unique.

In addition to the differences in structure between FcγRI and FcγRII these receptors differ in their specificity and affinity for IgG. Fc RI binds only mouse IgG2a with high affinity whereas FcγRII binds mouse IgG1, IgG2a and IgG2b.

Thus in vitro mutagenesis was used to determine functional regions of these FcγR and to generate chimeric FcγR with the combined properties of these receptors.

In addition in vitro mutagenesis was performed to generate hybrid FcR composed of functional regions of FcγRII and FcεRI wherein such a hybrid RcR possess the properties of both FcγRII and FcεRI.

Accordingly the invention provides a hybrid FcR molecule capable of binding to any one or more classes of antibody molecules.

Preferably the hybrid FcR comprises one or more functional domains of FcR linked to the structural or functional region of another molecule.

Preferably the hybrid FcR molecule binds to one of antibody classes IgM, IgG, IgA, IgD or IgE.

Preferably the hybrid FcR molecule is in soluble form.

Preferably the hybrid FcR molecule contains domains D1 and D2 of FcγRI linked to the transmembrane region and cytoplasmic tail of FcγRII and is designated FcγRI/II.

Preferably the hybrid FcR molecule contains domain d1 and d2 of FcγRII

linked to domain D3, transmembrane region and the cytoplasmic tail of Fc RI and is designated FcγRII/I.

Preferably the hybrid FcγR is human FcR.

Preferably the hybrid FcγR is mouse FcR.

Preferably the IgE binding region of IgE FcR is linked or located within FcγRI.

Preferably the IgE binding region of IgE FcεR is linked or located within FcγRII.

The invention further provides a hybrid FcR wherein at least one functional region comprises an extracellular domain, or parts thereof, derived from FcγRII having the following amino acid sequence :-

- Phe Ser Arg Leu Asp Pro Thr Phe Ser Tle Pro Gln Ala Asn His Ser His
Ser Gly Asp Tyr His Cys Thr Gly Asn Ile Gly Tyr Thr Leu Phe Ser Ser Lys
Pro Val Thr Ile Thr Val, or functional equivalent thereof.

The invention further provides a hybrid FcR wherein at least one functional region comprises an extracellular domain, or parts thereof, derived from FcεRI having the following amino acid sequence :-

- Trp Leu Leu Leu Gln Ala Ser Ala Glu Val Val Met Glu Gly Gln Pro Leu
Phe Leu Arg Cys His Gly Trp Arg Asn Trp Asp Val Tyr Lys Val Ile Tyr
Tyr Lys Asp Gly Glu Ala Leu Lys, or functional equivalent thereof.

Preferably the FcR is soluble or membrane bound.

The invention further provides:

A hybrid FcR being a chimera comprising a first extracellular domain of FcγRII, a second extracellular domain of FcεRI and a transmembrane region and cytoplasmic tail of FcγRII;

A hybrid FcR being a chimera comprising a first extracellular domains of FcεRI containing amino acids 1 - 169 of FcεRI linked to amino acids 170-281 of FcγRII;

A hybrid FcR being a chimera comprising a first extracellular domain of FcεRI being amino acids 1-86 linked to a second extracellular of FcγRII being amino acids 87-169 linked to a transmembrane region and cytoplasmic tail of FcγRII being

amino acids 170-281;

A hybrid FcR being a chimera comprising a first extracellular domain of FcεRI containing amino acids 1-86 linked to part of the second extracellular domain of FcεRI being amino acids 87-128 linked to part of the second extracellular domain of FcγRII being amino acids 129-169 linked to a transmembrane region and cytoplasmic tail of FcγRII being amino acids 170-281;

A hybrid FcR being a chimera comprising a first extracellular domain of FcεRI being amino acids 1-86 linked to part of the second extracellular domain of FcγRII being amino acids 87-128 linked to part of the second extracellular domain of FεRI being amino acids 129-169 linked to a transmembrane and cytoplasmic tail of FcγRII being amino acids 170-281;

A hybrid FcR being a chimera comprising a first extracellular domain of Fc RII being amino acids 1-86 linked to part of the second extracellular domain of FcγRII being amino acids 87-128 linked to part of the second extracellular domain of FcεRI being amino acids 129-169 linked to the transmembrane and cytoplasmic tail of FcγRII being amino acids 170-281;

A hybrid FcR being a chimera comprising the first extracellular domain of FcγRII being amino acids 1-86 linked to the second extracellular domain of FcεRI being amino acids 87-169 linked to a transmembrane and cytoplasmic tail of FcγRII being amino acids 170-281;

A hybrid FcR being a chimaera comprising the first extracellular domain of FcγRII being amino acids 1-86 linked to part of the second extracellular domain of FcεRI being amino acids 87-128 linked to part of the second extracellular domain of FcγRII being amino acids 129-169 linked to the transmembrane and cytoplasmic tail of Fc RII being amino acids 170-281.

The invention further provides amino acid and nucleotide sequences for hybrid FcR, polyclonal and monoclonal antibodies raised thereto, biological products incorporating FcR hybrids, diagnostic kits and assays and pharmaceuticals.

The invention further provides a method of generating hybrid FcR molecules comprising linking different functional domains of different Fc receptor molecules to construct hybrid FcR molecules.

The invention further provides a method of generating hybrid FcR molecules comprising ligating a first FcR molecule at the transmembrane junction, attaching a second FcR molecule to excise suitable domain regions and linking these two fragments to construct a hybrid Fc receptor molecule.

To generate these receptors an Apa-I restriction site was introduced into the cDNA sequence of FcγRI at the junction of sequences encoding D2 and D3. FcγRII cDNA already has an Apa site at the junction of d2 and the membrane spanning region. Thus the introduction of this restriction site (or any convenient restriction site) would facilitate the exchange of sequences between these or other receptors.

To introduce the Apa I site into the FcγRI cDNA two separate PCR mutagenesis reactions were performed and each used oligonucleotide primers containing an Apa I recognition sequence (Fig. 1) (13-15). The template for PCR was the FcγRI cDNA cloned into the pGEXII expression vector (7). PCR amplification of sequences FcγRI D1 and D2 was primed using a oligonucleotide containing Sal I site that hybridized to the leader sequence at the 5' end of the cDNA and the second primer MDHI which hybridized between D2 and D3 and would introduce an Apa I site between nucleotides 591 and 592 by altering three nucleotides of the FcγRI sequence GAGCTC to GGGCCC (Fig. 1). The second PCR amplified the sequence containing D3, the transmembrane domain and cytoplasmic tail. A primer (MDH2) also containing an Apa I recognition sequence hybridizes to the sequences between D2 and D3 of FcγRI in combination with the second oligonucleotide (MDH4) which contained a Sal I site. This primer hybridizes to the pGEXII vector sequences at the 3' end of the FcγRI cDNA insert.

The PCR products were then digested with Apa I or Sal I and ligated into FcγRII expression systems as outlined below.

To construct expressable FcR chimeric cDNA, Fc RII cDNA was subcloned into the PstI site of the expression vectors pKC3 or pKC4 downstream of the SV40 early promoter. These vectors differ only in the orientation of the polylinker and provided a unique Sal I site at the 5' or 3' end of the FcγRII cDNA (Fig. 2a). The pKC3-FcRII and pKC4-FcRII cDNA were digested with Sal I and Apa I to remove FcγRII d1 and d2 or the transmembrane and cytoplasmic encoding regions from these

vectors (Fig. 2b, 2c). The PCR products containing Sal I and Apa I sticky ends and encoding D1 and D2 of Fc γ RI (generated above; (Fig. 1)) were then subcloned into Sal I/Apa I digested pKC3-Fc γ RII. Similarly, to generate a hybrid cDNA encoding d1 and d2 of Fc γ RII linked to D3, and transmembrane and cytoplasmic tail of Fc γ RI. The PCR products containing D3, the transmembrane and cytoplasmic regions of Fc γ RI were linked to the D1 and D2 of Fc γ RII in the pKC4-Fc RII (Fig. 2e).

The chimeric cDNA were checked for the correct sequence by nucleotide sequencing. As expected the Fc γ RI/II chimera contained Fc γ RI nucleotide sequence from positions 9 to 594 that encodes Fc γ RI D1 and D2 and Fc γ RII cDNA sequence from positions 595 to 1244 that encode the transmembrane and cytoplasmic region of Fc γ RII (Fig. 3a). The Fc γ RII/I chimera contained Fc γ RII nucleotide sequence from positions 1-662 (encoding d1 and d2) and Fc γ RI sequence from positions 663-1348 that encode D3, the transmembrane and cytoplasmic tail of Fc γ RI (Fig. 3b).

The specificity of the chimeric Fc γ R and Fc γ RI and Fc γ RII were then tested in a transient expression system by transfecting the cDNA clones into monkey COS cells. The binding of rabbit IgG and mouse Ig subclasses was then assessed by EA rosetting. As expected rabbit IgG bound to cells transfected with Fc γ RI (Figure 4a) and Fc γ RII (Figure 4b) and also bound to both chimeric Fc γ R i.e. Fc γ RI/II (Figure 4c) and Fc γ RII/I (Fig. 4d, Table 1). Thus the D1 and D2 of Fc γ RI (now anchored in the cell membrane by the membrane spanning region of Fc γ RII) have the capacity to bind IgG in the absence of D3 of Fc γ RI.

Similarly, d1 and d2 of Fc γ RII retain the Ig binding capacity when associated with domain 3 of Fc γ RI (Fig. 1b, Table 1). Since Fc γ RI and Fc γ RII differ in their specificity for mouse IgG subclasses (Fc γ RI binds IgG2a but Fc γ RII binds IgG1, IgG2a, IgG2b (ref 1-10)) it was of interest to determine the specificity of the chimeric Fc γ R molecules for mouse IgG subclasses. As expected cells transfected with the native Fc γ RI bound IgG2a sensitised erythrocytes (Figure 4f) but did not bind IgG1 (Figure 4g) and cells transfected with vector only did not bind IgG-EA of any class (Figure 4h, Table 1). The Fc γ RI/II chimera (with the extracellular region consisting only of D1 and D2 of Fc γ RI) not only bound IgG2a (Figure 4i) but also

bound IgG1 (Figure 4j) and IgG2b (Table 1), which do not bind to native FcγRI (Table 1; refs 1-10) i.e. these FcγRI domains have now lost their unique specificity for IgG2a and IgG2b and have acquired the specificity of FcγRII. Thus, the specific binding of IgG2a by normal FcγRI involved D1 and D2 as well as D3 since the removal of D3 (by generation of the chimeric FcγRI/II) results in a broadening of the specificity of these domains. Thus by engineering the cDNA we were able to generate receptors of altered specificity i.e. convert FcγRI to FcγRII-like function.

Examination of the chimeric FcγR (FcγRII/I) containing d1 and d2 of FcγRII linked to D3, the transmembrane and cytoplasmic region of FcγRI, showed that this molecule bound IgG1, IgG2a and IgG2b, i.e. the specificity of d1 and d2 of FcγRII was retained. Furthermore, the presence of D3 of FcγRI did not influence the binding of the various mouse IgG subclasses to this chimeric FcγR.

The chimeric FcR proteins expressed on the surface of transfected cells were tested for reactivity with the 2.4G2 monoclonal antibody which reacts with FcγRII but not with FcγRI.

It is known that this antibody completely inhibits Ig binding to FcγRII and does not bind to FcγRI.

Transfected cells expressing normal and chimeric FcγR were pretreated with Fab fragments of the monoclonal antibody 2.4G2 and then tested for their ability to bind rabbit IgG coated erythrocytes. The Fab fragments completely inhibited binding of antibody coated erythrocytes to FcγRII and the chimeric FcγRII/I (Table 2). In addition, the 2.4G2 Fab₂ did not inhibit binding to FcγRI but by contrast completely inhibited binding to the chimeric FcγRI/II containing D1 and D2 FcγRI (Table 2). Thus D1 and D2 of FcγRI now express the 2.4G2 when not associated with D3 of FcγRI.

To further define the role of the FcγRI extracellular domains, the binding of monomeric IgG to the chimaeric receptors was investigated. The affinity of monomeric IgG binding was assessed by Scatchard analysis with ¹²⁵I labelled monoclonal antibodies of IgG2a subtype on transfected COS cells expressing either of the two chimaeric receptors, native FcγRI or FcγRII. The high affinity receptor FcγRI bound IgG2a with affinity $1.4 \times 10^8 \text{M}^{-1}$ (Figure 5) which is similar to that

reported previously (1-3,7). In contrast, the binding of monomeric IgG2a by the FcγRI/II and FcγRII/I chimaeric receptors, and the low affinity receptor FcγRII (as expected), was not detectable (not shown).

Furthermore, high affinity binding was not able to be generated by linking D3 of FcγRI to d1 and d2 of FcγRII, despite the high homology of FcγRII d1-d2 with FcγRI D1-D2. This suggests that all three extracellular domains of FcγRI are required for the high affinity interaction with IgG2a, D3 being crucial in modifying the D1-D2 interaction with IgG binding properties of FcγRII, as they do not detectably bind monomeric IgG2a and also show the broader specificity for IgG1, 2a, 2b immune complexes that is characteristic of FcγRII. Clearly these domains represent a conserved FcγRII-like IgG binding unit between these otherwise functionally distinct receptors. However, there must be specialised differences between the homologous two domain structure of FcγRI and FcγRII, as only D1 and D2 of FcγRI, not d1 and d2 of FcγRII, have the capacity to interact with D3 to produce the specific high affinity binding of IgG2a.

It should be noted that the strategy used to generate the FcγRII/I chimaeric cDNA led to the replacement of the glutamic acid and leucine residues at positions 172 and 173 with glycine and proline, through the introduction of an Apal site at the junction of D2 and D3 of FcγRI. To ensure that these amino acid substitutions were not responsible for the observed properties of the FcγRII/I chimaeric receptor, mutagenesis was used to replace the glycine and proline residues with the original glutamic acid and leucine residues. This chimaeric receptor exhibited identical IgG binding characteristics to the original FcγRII/I chimaera (data not shown).

Based on findings presented herein, it is possible to make several statements on the origin of FcγRI which highlight important aspects regarding evolution of FcγR genes and the Ig superfamily. Given that there is considerable amino acid homology between D1-D2 of FcγRI and d1-d2 of FcγRII(7), and that D1-D2 adopts the specificity and affinity for IgG of FcγRII after the removal of D3, it is clear that the two receptors have a common evolutionary history. Indeed, the recent cloning and mapping of multiple mouse and human FcγRII genes, multiple human FcγRI genes and FcγRIII genes, and both the mouse and human FcεRI genes, indicate there has

been considerable gene duplication in the same region of chromosome 1 to produce this subfamily of Ig related molecules which show great amino acid homology (16-19).

The substantial amino acid identity between the extracellular regions of IgG receptors, (approximately 90% within a class e.g. FcγRIIa as compared to FcγRIIb1, FcγRIIb2 and FcγRIIb3 or FcγRIII-1 compared to FcγRIII-2) or between the extracellular regions between FcR classes (e.g. approximately 50% between FcγRI and FcγRIII and 40% between FcγRII and FcεRI) indicates shared structure although there are clear differences in function or specificity (4-12). This high conservation of amino acid identity between FcR is also seen between the low affinity IgG FcγRII and the high affinity IgE receptor FcεRI. Although these receptors have very distinct specificity for immunoglobulins, FcγRII binds IgG whereas FcεRI binds IgE, these FcR share approximately 40% amino acid identity in their Ig binding, extracellular domains.

Thus it should be possible to generate many novel chimaeric Fc receptors that contain parts and therefore characteristics and functions of multiple receptor classes e.g. a chimaeric FcR composed of FcγRII and FcεRI sequences that binds IgG and IgE; a chimaeric FcγR composed of parts of FcγRII and FcγRI or FcγRII and FcγRIII, or FcαR (IgA receptors) and FcγRII or other FcR. Similarly these chimeric receptors may be composed of sequences derived from three or more Fc receptors e.g. FcγRII and FcγRI and FcεRI or FcγRI and FcαR and FcεRI and FcμR (IgM receptors) etc.

The origin of the third domain in the extracellular region of FcγRI is also of interest, unlike FcγRII and FcγRIII, FcγRI is unique in that it has an additional extracellular domain. Whether D3 of FcγRI arose by duplication of a related exon or by a insertion of an exogenous exon is not clear, as sequence comparisons indicate D3 is only distantly related to D1 and D2, or to domains of other Ig superfamily members. However the data presented herein demonstrates how Ig-superfamily genes may have evolved, in that FcγRI probably originated through the acquisition of an exon encoding an intact Ig like domain (D3), presumably by a primordial FcγRII-like gene, with subsequent divergence refining the interaction of the three

domains to produce a new receptor with modified IgG binding characteristics i.e. a specialised high affinity receptor for IgG2a. This model for FcγRI evolution is consistent with the proposed evolution of the Ig superfamily postulated by Williams and colleagues, wherein primordial genes acquire individual exons encoding Ig-like domains, and these domains become functionally incorporated to create new molecules with new function. Indeed, each of the extracellular Ig-like domains of mouse FcγRI and FcγRII are encoded by individual exons (N. Osman, M. Hogarth unpublished observations, Ref. 19).

Finally, based on the high homology between D1, D2 and D3 of human and mouse FcγRI (69,74 and 75% amino acid identity respectively (7), together with the observation that human FcγRI also binds mouse IgG2a with high affinity (10), it is likely that the contributions of the extracellular domains of human FcγRI to IgG binding will parallel those reported here for mouse FcγRI and FcγRII.

Clearly it is possible to generate functional chimeric FcR and several statements can be made. First chimeric cDNA clones encoding FcR composed of components of different FcγR have been generated. Second, by connecting D1 and D2 of FcγRI to the transmembrane cytoplasmic regions of FcγRII a receptor molecule was produced that has a broader specificity than the receptor from which the Ig binding regions were derived, i.e. FcγRI/II containing D1 and D2 of FcγRI binds mouse IgG1, IgG2a, IgG2b. Thirdly, similar experiments that generate chimeric FcγR between human FcγRII and human FcγRI have shown that these chimeric receptors have altered specificity for IgG. Fourthly, it is possible to generate numerous other chimeric FcR that possess characteristics of other FcR. Since most FcR are homologous proteins it is possible to insert/or attach Ig binding regions to FcγRI or II e.g. insert the IgE binding region of IgE FcR (FcεRI) into another FcR e.g. FcγRII.

Indeed, the following experiments describe the production of chimaeric receptors containing amino acid sequence elements of the human low affinity FcγRII and the human high affinity IgE receptor FcεRI. These chimeras were generated by exchanging parts of the cDNA sequence encoding the regions shown in the Figures 6,7 and Tables 3,4. Although the IgG and IgE receptors have distinct biological

properties and specificities they are homologous transmembrane glycoproteins, both containing two extracellular immunoglobulin binding domains (EC domains) which have approximately 40% amino acid identity. This considerable identity presumably indicates that the extracellular portions of these distinct molecules would be expected to have a similar general structure. Chimeric receptors formed between these classes of receptors would therefore be expected to be expressed on the cell surface, processed appropriately and indeed the transfection experiments indicate that this is the case (Figure 8, Table 5). We have successfully isolated and transfected the human Fc γ RII cDNA defined in Australian Patent Serial No. 595623 (reference 6) and the cDNA sequence is shown in Figure 6. The human high affinity receptor for IgE has been previously described (12) and the sequence is shown in Figure 7. This receptor is expressed extensively on mast cells and basophils and shows a high affinity for IgE. Biochemical studies have indicated that the Ig binding subunit (α subunit) (sequence shown in Figure 7) is associated with two additional subunits, the β subunit and a dimer of two γ subunits that are disulphide bonded to one another. Expression of the α subunit normally requires the expression of one or more of the β or γ subunits (21). The strategy employed to examine the Ig binding characteristics of the extracellular domains of Fc γ RII and Fc ϵ RI was to exchange the entire domains or parts of domains between two types of receptors and examine the specificity of these receptors with respect to the binding of IgG and IgE. The nucleotide sequence of all chimeric cDNA described herein (Table 3,4) was confirmed by nucleotide sequencing.

Since the Fc ϵ RI subunit requires the presence of additional subunits for expression and the subunits are not synthesised in the COS cells (which are used as the target cells for transfection and expression experiments) an Fc ϵ RI α chain capable of being expressed alone was generated. The initial strategy involved placing the entire extracellular domains of Fc ϵ RI on the membrane spanning and cytoplasmic region of Fc γ RII. This was performed by splice overlap extension (SOE) using oligonucleotides EG6 and EG1 (Table 4) which hybridise to the Fc ϵ RI cDNA (Figure 7) to generate amplified Fc ϵ RI sequence and the oligonucleotide pair EG2 and EG5 (Table 4) which hybridise to the Fc γ RII cDNA (Figure 6) to generate the amplified

Fc γ RII sequence. Since sequence within EG1 and EG2 overlap, the two amplified products were used in the overlap extension to produce the full length recombinant Chimaera 1 (Table 3). Thus a chimaeric cDNA was generated which encoded the 25 amino acids of the Fc ϵ RI leader sequence and 169 amino acids of the extracellular portion of Fc ϵ RI which was linked to the amino acid sequences from position 170 in Fc γ RII to the C terminal amino acid as position 281 of Fc γ RII (Table 3 and 4).

To establish if this chimeric cDNA encoded a functional IgE receptor the chimeric cDNA contained in expression vector pKC3 (Van Doren et al., J. Virol. 50:606, 1984) was transfected into COS cells in a transient expression system and expression measured 48-72 hours later. Expression was tested by EA rosetting using IgE sensitised erythrocytes and by a direct binding assay using Scatchard analysis using 125 iodine labelled human IgE. Cells transfected with the Chimaera 1 cDNA bound IgE immune complexes, [IgE sensitized erythrocytes (Figures 8a)] and also bound monomeric human IgE with high affinity, but did not bind IgG sensitised erythrocytes (Figures 8d, Table 5) as would be expected of an Fc ϵ RI. Conversely, Fc γ RII bound IgG-EA (Figure 8b) but not IgGE-EA (Figure 8A) as expected. This experiment establishes that the expression of Chimaera 1 and the interaction with and specificity for IgE of the Ec domains of Fc ϵ RI are not dependent on the additional subunits or the membrane spanning region of Fc ϵ RI.

In the light of experiments using FcR, with the high and low affinity IgG receptors (described earlier) a series of chimeric cDNAs and Fc receptor proteins containing Fc γ RII and Fc ϵ RI sequences were then produced in order to:-

1. Establish the extent to which replacement of extracellular sequences from one receptor with those of a second receptor would enable the production of functional Fc receptors.
2. Generate functional receptors that have properties of multiple Fc receptors e.g. Fc receptors that bound both IgG and IgE.
3. Localise the regions of Fc receptors responsible for the interaction with immunoglobulins.

To determine the functional Fc binding domain(s) of Fc ϵ RI and Fc γ RII, chimaeric receptors were generated such that they contained two extracellular

domains (EC domains), one of which was derived from FcεRI, the second of which was derived from FcγRII (Table 3,4,5). Chimera 2 contained the first extracellular domain of FcγRII and the second domain of FcεRI organised in that order. This chimera was generated by splice overlap extension (SOE) using the oligonucleotides NR1 and EG11 as well as EG10 and EG5 to respectively amplify the appropriate sequences from FcγRII cDNA or from the Chimera 1 cDNA (Table 4, Figures 6,7). The overlap extension reactions were performed as detailed in the Materials and Methods and were possible because of the overlapping sequence contained in oligonucleotides EG10 and EG11. The resulting chimaeric cDNA (Chimera 2) encoded a chimaeric Fc receptor containing the FcγRII leader sequence (amino acid-34 to the N-terminus) and the first EC domain of FcγRII amino acid 1 to 86 inclusive, constituting the first EC domain of the Chimera 2. In addition the overlap extension reactions also included the second EC domain of FcεRI corresponding to amino acids 87 to 169 of this receptor. As Chimera 1 was used as a template for this region, Chimera 2 also contains the membrane spanning region and cytoplasmic tail of FcγRII, which included amino acids 170 to 281 (Table 3). Similarly, the chimaeric Fc receptor (Chimera 3) was generated to contain the first EC domain of FcεRI and the second EC domain, transmembrane region and cytoplasmic tail of FcγRII. Splice overlap extension was used to generate the chimeric cDNA that encoded this receptor by using oligonucleotide pair EG6 and EG9 on the FcεRI cDNA template and oligonucleotides EG8 and EG5 with FcγRII cDNA as a template. The splice overlap extension reactions were possible because of the overlapping sequence contained in oligonucleotides EG9 and EG8. The Fc receptor encoded by this chimeric cDNA contained the leader sequence (amino acids-25 to -1 of FcεRI) as well as the first EC domain of FcεRI (amino acids 1 to 86) as well as the amino acids of the second EC domain, membrane spanning and cytoplasmic tail of FcγRII (amino acids 87 to 281 inclusive) (Tables 3,4 Figures 6,7).

To assess the specificity of these chimaeric receptors, the chimaeric cDNAs, cloned into the pKC3 expression vector, were transiently expressed by transfection into COS 7 cells. The capacity of these Fc receptors expressed on the transfected cells to bind IgG or IgE was tested by the binding of immune complexes in the form

of erythrocytes sensitised with IgG or IgE (IgG-EA or IgE-EA). Chimaera 2 containing the first EC domain of FcγRII and the second EC domain of FcεRI bound IgE but did not bind IgG sensitised erythrocytes (Figure 8e,f; Table 5). Conversely, Chimaera 3 containing the first EC domain of FcεRI and the second EC domain of FcγRII bound IgG but not IgE immune complexes (Figure 8g,h). These results indicate that the second EC domain is intimately involved in the interaction with immunoglobulins since in Chimaera 2 the first EC domain was derived from the IgG receptor and the second from the IgE receptor but only bound IgE not IgG. Conversely, in Chimaera 3 the first EC domain was derived from the IgE receptor and the second from the IgG receptor and could clearly bind IgG but not IgE. Since the normal Fc RII and normal FcεRI receptors exclusively bind IgG and IgE respectively (as does Chimaera 1) it is clear that the interaction of Chimearas 2 and 3 with IgE and IgG respectively is mediated within the second EC domain of Fc receptors (Table 3).

To localise the binding of immunoglobulins to a subregions of EC domain 2 in both FcγRII and FcεRI additional chimaeric receptors were generated wherein EC domain 1 was derived from either FcεRI and FcγRII (Table 4). These chimeric receptors were generated using the splice overlap extension technique, where amplification of the appropriate regions of cDNA were performed on the wild type FcεRI or FcγRII cDNA as well as the appropriate chimaeric cDNA (Table 3, 4). Chimaera 4 contained the first EC domain of FcεRI together with a substantial amino terminal portion of the second EC domain of FcεRI (amino acid -25 to -1 together with amino acids 1 to 128 inclusive). These amino acids were connected to the C-terminal portion of the second EC domain of FcγRII, and membrane/ cytoplasmic regions of FcγRII amino acids 129 to 281 inclusive (Table 3). This chimaeric cDNA was generated using the oligonucleotides EG6 with EG14 on the FcεRI cDNA template, and EG15 together with EG5 on the FcγRII cDNA template (Table 4). COS cells were transfected with this chimaeric cDNA and the binding of IgE and IgG to the encoded Chimaera 4 receptor was examined by the binding of immune complexes in the form of antibody sensitized erythrocytes (Figure 8i, j; Table 5). These experiments indicated that the chimaeric Fc receptor Chimaera 4 bound both

IgG-EA and IgE-EA, this indicated that an IgG binding site could be localised to the C-terminal portion of the second EC domain within the region encoded by amino acids 129 to 169 inclusive. The observation that Chimaera 4 bound IgE-Ea also indicates that an IgE binding region is located in the second domain in the region containing amino acids 87 to 128 inclusive. The involvement of these regions in the binding of IgE and IgG was verified by the construction of Chimera 5 and its subsequent transfection and immune complex binding studies.

Chimera 5 was generated by the splice overlap extension techniques using the oligonucleotides outlined in Table 4. This chimera contained the first extracellular domain of FcεRI with the second EC domain composed of both FcγRII (amino acids 87 to 128 inclusive) and FcεRI sequence (amino acids 129 to 169). Transfection of this cDNA indicated that the chimaeric Fc receptor failed to bind IgG or IgE (Tables 3 and 5). The amino acid sequence of Chimeras 4 and 5 differs only in the amino acid sequence within the second EC domain. Wherein amino acid residues, 87 to 128 inclusive, of Chimaera 4 have been derived from FcεRI and have been replaced with residues 87 to 128 of FcγRII in Chimaera 5. Since Chimaera 4 binds IgE and Chimaera 5 does not, residues (87-128) in FcεRI are important in IgE binding. Similarly as Chimaera 4 contains amino acid residues 129 to 169 inclusive, derived from FcγRII and binds IgG but Chimaera 5 which contains amino acids 129 to 169 inclusive, derived from FcεRI and does not bind IgG, indicates that these residues are important in IgG binding. Since Chimeras 4 and 5 are half domain chimeras ie. containing sequence from FcεRI and FcγRII within the second domain but have the same first EC domain derived from FcεRI, two additional chimeras were made again containing the same amino acid configurations of the second EC domain as found in chimeras 4 and 5, but containing the first EC domain derived from FcγRII. Chimaera 6 contains the first EC domain of FcγRII including the leader sequence corresponding to amino acids -34 to 1 and amino acids 1-86 inclusive as found in the first EC domain of FcγRII (Table 3, Figure 6). This was attached to a second EC domain wherein amino acids 87 to 128 inclusive were derived from FcεRI and amino acids 129 to 169 inclusive were derived from FcγRII (Figures 6,7). Transfection of this cDNA into COS cells showed that the encoded chimaeric

receptor could bind IgG and IgE (Table 3). By contrast, Chimaera 7 which contained the EC domain 1 of Fc γ RII but contained amino acids 87-128 of Fc γ RII and amino acids 129 to 169 of Fc ϵ RII but contained amino acids 87-128 of Fc γ RII and amino acids 129 to 169 of Fc ϵ RI failed to bind IgG or IgE when transfected into COS cells (Table 5). These results taken together with those experiments using the Chimeras 4 and 5 indicate that amino acids within the second domain are clearly important in the interaction of Fc receptors with Ig, and particularly amino acids 87-128 of Fc ϵ RI are important in the interaction with IgE and amino acids 129 to 169 of Fc γ RII in the interaction with IgG.

To further characterise the chimaeric receptor structures, monoclonal anti Fc γ RII antibodies (description of antibodies described below) were used in immunofluorescence studies on cells transfected with native Fc γ RII or chimaeric cDNAs. All monoclonal anti Fc γ RII antibodies bound to cells transfected with the native Fc γ RII cDNA (Table 5). As expected none of these antibodies bound to cells transfected with the native Fc ϵ RI α subunit or cells transfected with Chimaera 1, which contains the extracellular IgE binding domains (Table 5).

Analysis of monoclonal antibody (MAb) binding to the single domain chimaeras indicated that Chimaeras 2 and 3 have some obvious structural differences (Table 5). Monoclonal antibodies 8.7 and 7.30 detected the expression of Chimaera 3 but did not bind to Chimaera 2. Since Chimaera 3 contains EC domain 1 from Fc ϵ RI and EC domain 2 from Fc γ RII, this result indicates that the epitope on Fc γ RII molecules detected by 8.7 and 7.30 (see below) is located in EC domain of Fc γ RII. By contrast, monoclonal antibodies 8.2 and 8.26 which bound to the native Fc γ RII failed to bind to any of the chimaeric receptors tested (Table 5), indicating that this epitope detected by these antibodies requires the presence of part or all the first and second EC domains and this clearly establishes the epitope detected by 8.2 and 8.26 as distinct from the epitope detected by antibodies 8.7 and 7.30. Of interest was the finding that the previously described (22) monoclonal antibody IV.3 bound to Chimaera 3, whereas a second previously described antibody CIKM5 (23) did not bind to any chimaeric cDNA. However, both antibodies bound to the native Fc γ RII.

The construction of these chimaeric receptors and the immune complex

binding studies as well as the characterisation using monoclonal antibodies has indicated several clear features of these receptors:-

- 1) The EC domains of FcεRI can be attached to the membrane spanning and cytoplasmic regions of a different molecule (FcγRII) and the binding of IgE to the extracellular domains of FcεRI is retained i.e. the extracellular domains function as receptors irrespective of additional sequence added at the C-terminal end of the EC domains. Clearly soluble forms of this receptor i.e. a form of the receptor containing only the extracellular domains or part thereof in the absence of a membrane spanning segment or cytoplasmic tail, would be expected to bind IgE. Furthermore, the chimaeric receptors which contained appropriate FcεRI or FcγRII sequences (e.g. as found in Chimaeras 4 and 5) would also be expected in a soluble form to bind IgG and/or IgE.
- 2) The second EC domain, EC domain 2, is intimately involved in the binding of IgE and IgG. Indeed, the binding of these immunoglobulins to Chimera 4 and Chimera 6 indicate that the amino acids that correspond to residues 87 to 128 inclusive of FcεRI are intimately involved with IgE binding and residues corresponding to amino acid residues 129 - 169 of FcγRII are intimately involved in IgG binding (Tables 3-5, Figure 8). These results identify these residues of the domain 2 as being important in the binding of IgE and IgG but do not exclude other regions of the receptor also being intimately involved.
- 3) It is clearly possible to construct multi-functional chimaeric Fc receptors that have properties of several different receptor types. Such multi-functional receptors would be expected to have advantages over individual receptor classes in that the production of a single chimaeric form of Fc receptor would have the functions of multiple Fc receptor classes and would circumvent the need for the production of two individual Fc receptor types with subsequent mixing of these receptors in any pharmaceutical preparation or assay system. The uses of these chimaeric receptors in the diagnosis and treatment of allergy, autoimmune disease, parasite infections, immune complex disease and a range of haemopoietic and non-haemopoietic disorders would be

significantly more straight forward, than using single function receptors.

- 4) Thus the manipulation of FcR will enable the construction of novel chimaeric FcR that can be high or low affinity and bind immunoglobulin of multiple classes. Receptors will have domain or subdomain sequences derived from different receptors (e.g. Ig binding domain or sequences of FcεRI or high affinity domains (D3) of FcγRII that are intimately involved in a particular functions resulting in a single receptor type having multiple functions).

In addition, these functional regions could be attached to non FcR molecules e.g. antibody molecules, or bacterial proteins e.g. to create fusion proteins, wherein FcR function is maintained in addition to the function of the non FcR protein.

- 5) Furthermore, the monoclonal antibody experiments indicated that the EC domain structure is sufficiently conserved in some case eg. Chimaera 3, and not only interacts with Ig but retains the epitope detected by the monoclonal antibodies 8.7 and 7.30. However, some alteration to the total overall structure is apparent since the epitope detected by antibody 8.2 and 8.26 has been lost from Chimaera 3 as a result of the construction of the chimaeric receptors containing sequences from the FcεRI.

The studies described below of the characterisation of these monoclonal anti FcγR antibodies identify antibodies 8.2, 8.26, 8.7, 7.30 as unique monoclonal antibody which are embodied in this patent application.

The characterisation of the native and chimaeric FcR was aided by the use of monoclonal antibodies (MAb). These antibodies recognise cell surface FcγR and also soluble circulating FcR. This has been made possible by the production and characterisation of 4 new MAb (designated 8.2, 8.7, 8.26, 7.30) recognising human FcγRII is described and the use of these MAb in the detection of soluble FcγRII (sFcγRII) in a two antibody radioimmunoassay.

Four MAbs were derived by cell fusion for characterisation. Three MAb [8.2 (of the IgG1 subclass), 8.7 (IgG1) and 8.26 (IgG2b)] were produced by immunisation of BALB/c mice with K562 cells. A fourth "second generation" MAb 7.30 (IgG1) was produced by immunisation with FcγRII 3.0 cDNA transfected L-cells. Cell fusions were performed as described in the Materials and Methods.

Tissue Distribution by FACS Analysis.

The tissue distribution of the antigens detected by the MAb was determined by quantitative analysis of fluorescence staining of FcγRII 3.0 transfected L-cells (Tf3.0) and a number of haemopoietic cell lines. This analysis shows that the four new MAb have strong specific positive reactions with FcγRII 3.0 cDNA transfected L-cells, similar to the two CDW32 reference MAb, IV.3 and CIKM5 described by others (22, 23) which are included for the purposes of comparison. The tissue distribution of the new MAbs on the haemopoietic cell lines have profiles which distinguish these from each other and from IV.3 and CIKM5. All antibodies bound strongly to K562 cells but had unique binding on Daudi cells where only MAb 8.7 and 7.30 bound strongly to Daudi (Table 6). The MAb 8.26 bound less strongly and antibody 8.2 showed very weak binding as did the IV.3 and CIKM5 antibodies. Weak binding to Raji cells was also obtained with MAb 8.7, 8.26 and 7.30 and there was no detectable binding with the 8.2 antibody. All antibodies bound to an EBV transformed B cell line (Table 6). It should be noted that the MAb 8.26 gave consistently higher fluorescence on B cell lines (Daudi, Raji, EBV cells) than did the 8.2 and IV.3 MAb.

The binding to myeloid cells was also examined. Antibodies bound to the U937, Thp-1 and HL60 cell lines. However 8.7 and 8.26 could be distinguished from other MAb as they bound less strongly to U937 cells (Table 6). The fluorescence profiles of the binding of the MAb to Daudi and U937 cells is shown in Figure 9. Antibodies IV.3, CIKM5, 8.2 and 8.26 react with greater fluorescence intensity on U937 than on Daudi. Conversely antibodies 8.7 and 7.30 exhibit greater fluorescence than 8.2 or 8.26 or IV.3 and CIKM5. It should be noted that 8.26 has greater reactivity on all B cell lines and can be distinguished from 8.2, IV.3 and CIKM5 (Figure 9). All 6 MAb were negative on the 4 T-cell lines tested.

Inhibition of Fc Binding.

To determine the relationship of the epitopes detected by these antibodies to the Ig binding site, the capacity of these antibodies to inhibit immune complex (IgG-EA) binding was assessed (Table 7). Results of EA inhibition assays on three FcγRII

bearing cell lines is shown in Table 2. The three cell lines K562, FcγRII cDNA transfectants (Tf3.0) and Daudi cells express only FcγRII. The pattern of inhibition separates the 6 MAb into 3 groups; IV.3, 8.2 and 8.26 give 80–100% inhibition of EA rosetting on all 3 cell lines. The 8.7 and 7.30 antibodies completely inhibit EA rosetting on Daudi cells, but only partially inhibited EA rosetting on K562 and transfected L-cells. CIKM5 alone, gives partial inhibition of EA on K562 only.

Competitive Inhibition Assays.

To Determine Relationship Between Epitopes detected monoclonal antibodies. Competitive inhibition assays between the MAb were initially performed on K562 cells (Table 8) and on transfectants expressing FcγRII (Figure 10). When tested on the K562 cells radiolabelled IV.3, 8.2 and 8.26 show similar complete reciprocal inhibition patterns with unlabelled IV.3, 8.2 and 8.26 i.e. they completely inhibit each others binding indicating these MAb recognise overlapping epitopes. The epitope detected by the IV.3 antibody was unique, as unlabelled IV.3 (but not 8.2 and 8.26) completely inhibited the binding of labelled 8.7, 7.30 and CIKM5. Antibodies 8.7 and 7.30 formed a second distinct group as these completely inhibited each others binding to K562 cells indicating these detect identical epitopes. However, 8.2 or 8.26 did not inhibit the binding of 8.7 and 7.30. Unlabelled CIKM5 either failed to inhibit or only partially inhibited the binding of all other anti-FcγR MAbs tested (Table 8). As discussed below, these results taken together with the tissue distributions and EA inhibition studies indicate the presence of at least 4 epitopes:– Epitope 1 detected by the IV.3 antibody. Epitope 2 detected by the CIKM5 antibody. Epitope 3 by the 8.2 and 8.26 antibodies and Epitope 4 detected by antibodies 7.30 and 8.7.

The competitive binding experiments were also performed on FcγRII cDNA transfected L-cells which express a single form of FcγRII (Figure 10). One MAb defining each of the 4 possible epitopes was studied (IV.3, CIKM5, 8.2, 7.30). The results of competitive inhibition assays are shown in Figure 10. The studies with the Fc RII transfected cells confirms the pattern of inhibition seen when K562 cells were used as target cells.

Immunoprecipitation.

The Fab or Fab₂ fragments of the anti FcγRII MAb were used for

immunoprecipitation studies from cell lysates of surface labelled K562 cells or Daudi cells (Figure 11A,B). Antibodies IV.3, CIKM5, 8.2 and 8.26 precipitated protein from K562 cells which migrated as a broad band on an SDS-PAGE gel with a Mr of 40-43kd. No material was precipitated with 8.7 antibody. A similar 40-43kd was immunoprecipitated with IV.3, 8.2 and 8.26 MAb from Daudi cells. In addition, aggregated human IgG and a rabbit polyclonal anti-FcγRII antibody immuno precipitated material of identical Mr to that of the FcγRII precipitated by the monoclonal antibodies (Fig. 11A, B).

Assay for circulating soluble FcγRII.

Using a two antibody sandwich radioimmunoassay normal individuals and a series of patients with autoimmune disorders (Systemic Lupus Erythematosus [SLE], Rheumatoid arthritis and Sjogren Syndrome) were tested for serum levels of soluble FcγRII. The results of this assay indicate that soluble FcγRII may be detected in normal individuals and patients with autoimmune diseases have significantly higher circulating levels of soluble FcγRII. The present of higher levels of circulating FcγRII is most evident in serum from individuals with Sjogren's Syndrome. Elevated levels were also detected in the circulation of patients with rheumatoid arthritis or Systemic Lupus erythematosus.

The production and characterization of four monoclonal antibodies to human FcγRII is useful in the study of cellular and circulating FcγRII and its role or presence in disease. The four new monoclonal antibodies (8.2, 8.26, 8.7, 7.30) clearly detect human FcγRII. Since they:-

- 1) Bind specifically to mouse L cells transfected with human FcγRII cDNA but do not bind to the untransfected cells.
- 2) Show a tissue distribution that is identical to that expected for FcγRII.
- 3) Immunoprecipitate a 40 - 43 kd molecule that is the expected size of FcγRII and is the subject of Australian Patent Serial Number 595623.
- 4) All antibodies partially or completely inhibit the binding of immune complexes (IgG- EA) to FcγRII positive target cells.

Clearly these antibodies detect FcγRII molecules. However it is clear that the antibodies fall into two discrete groups which when taken together with experiments

using two previously defined monoclonal FcγRII antibodies, indicate that at least four epitopes exist on FcγRII. The groups defined thus:-

- (i) 8.2, 8.26
- (ii) 8.7, 7.30
- (iii) IV.3
- (iv) CIKM5

These antibody groups can be distinguished from each other on the basis of (i) tissue distribution (ii) differences in the capacity to inhibit the binding of immune complexes, EA rosettes - (CIKM5 can only weakly inhibits EA rosetting whereas all other antibodies completely or significantly inhibit rosetting) and (iii) competitive inhibition assays indicate that the antibodies in the groups shown above detect four distinct epitopes. These distinct epitopes may be generated on mature cell surface FcγRII by post translation modification, such that the epitopes are carbohydrate determined; alternatively the antibodies detect protein epitopes in the peptide core.

It is clear that multiple forms of FcγRII have been defined (4-6, 8). However, these different forms have highly homologous extra cellular regions, wherein the amino acid identity was greater than 90% and the nucleic and amino acid sequence of the EC domains of the FcγRII, b1 and b3 are in fact identical. This high conservation of amino acid identify also results in high conservation of FcγRII function, in that all Fc receptors identified have the capacity to bind IgG immune complexes. To determine if the monoclonal antibodies described herein (8.2, 8.26, 8.7, 7.30) can distinguish between extracellular sequences of these multiple isoforms, transfection experiments were performed wherein cells were transfected with human FcγRII a clone 3.0 or transfected with the human FcγRII b1 clone (8). All monoclonal antibodies, 8.2, 8.26, 8.7, 7.30 bound to both FcγRIIa and FcγRII b1 and since the amino acid sequence of EC portion (both domains) of Fc RII b1, b2, b3 is identical (8) these MAb would detect FcγRIIb2 and b3 also.

Since the antibodies 8.26 and 8.7 detect distinct non overlapping epitopes, a capture tag radio immunoassay for the detection of soluble Fc receptor was developed. One monoclonal antibody was attached to the PVC plate and used to capture circulating Fc receptor in the blood of normal or diseased individuals. A

second antibody was used to detect the captured Fc receptor, this second antibody was tagged (eg. radio- labelled) on such a way that binding of this antibody could be detected. As shown in Figure 12 the second antibody was radiolabelled with I 125 and the specific binding of radiolabelled antibody to captured Fc receptor indicated the presence of circulating Fc receptor in blood derived from different individuals. In this study three groups of patients with autoimmune disease were studied: SLE, RA and Sjogrens syndrome. When compared to normal individuals all groups of patients with autoimmune disease has statistically significant high levels of soluble FcγRII. It is clear that this assay can be used to identify circulating soluble receptor and the assay can be adapted to be performed with other monoclonal antibodies or polyclonal receptor antibody. The second antibody can also be radiolabelled or conjugated to a flurochrome or enzyme and be used in photometric or colormetric assays, e.g. ELISA assays.

The detection of circulating FcγR may be of diagnostic use for patients with autoimmune or other diseases where high levels of receptor are of diagnostic or prognostic value or as an indicator of disease activity. Indeed, the patients afflicted by Sjorgens Syndrome and the diseases indicated (SLE and RA) (Figure 12) have much higher levels of circulating receptor than normal individuals. Although the source of the circulating FcγRII is unknown it is clear that it can be detected in the circulation;. Furthermore, as all the MAb - detect all isoforms of FcγRII - i.e. FcγRIIa, FcγRIIb1 (and FcγRIIb2, b3) then the assay would also detect the circulating forms of these. However, the fact that the precise nature of the circulating FcγRII is unknown and does not compromise the use and application of the assay for the detection of soluble FcγRII.

The application of this assay for the detection of soluble Fc receptor is not restricted to use in assays of patient serum but is equally applicable for assays using other bodily fluids, eg. urine, saliva, synovial fluid, or faeces.

Concluding Remarks

We have demonstrated the principle that chimeric Fc receptors derived in such a fashion that they contain amino acid sequence from multiple Fc receptors can also retain the immunoglobulin binding functions of these receptors types. Also the

identification of the function or active region of these receptors (ie. immunoglobulin interactive) regions also identifies a useful region of these molecules. Monoclonal antibodies were used to demonstrate the preservation of epitopes in these chimaeric molecules and detect soluble Fc receptor in the circulation of diseased individuals.

MATERIALS AND METHODS

Molecular genetic techniques.

Standard molecular genetic techniques were performed as described in references 13 and 14 and included; restriction digestion, electrophoresis and purification of DNA fragments, ligation, bacterial transformation, di-deoxy nucleotide sequencing, plasmid DNA preparation, nucleic acid phosphorylation and de-phosphorylation, hybridisation, Southern blots.

Splice overlap extension (SOE).

Chimaeric cDNA (which encode chimeric Fc receptors) were produced by splice overlap extension (SOE) using three polmerase chain reaction (PCR) steps essentially as described (24,25). The first PCR was performed to amplify sequence that forms 5' section of the chimaeric cDNA. The second PCR amplified sequence that forms the 3' section of the chimeric cDNA. Oligonuleotides (500ng) hybridising to the 5' or 3' ends of the region to be amplified were mixed with the appropriate template (100ng) and 25 cycles were performed using a thermal cycler (see below). The sequence of oligonucleotides used in the PCR reactions were designed such that the oligonucleotide primer hybridising to the 3' end of the first PCR product (this makes up the 5' sequences of the chimeric cDNA) overlaps sequence contained within the oligonucleotide primer hybridising to the 5' end of the second PCR product (this makes up the 3' sequences of the chimaeric cDNA) (Figures 6,7 Table 3,4,9). Thus the two independently derived PCR products overlap at their 3' (PCR product 1) and 5' (PCR product 2) ends.

The splice overlap extension reaction (ie third PCR) generates the chimeric cDNA for subsequent manipulation and was performed using purified PCR products 1 and 2. Approximately 10ng of each of the PCR product was mixed with two oligonucleotide primers (used in PCR 1 and PCR 2) that hybridise to the 5' end of PCR product 1 or the 3' end of PCR product 2. Also the 5' oligonucleotides (NRI or

EG6) used in the third PCR contained a EcoRI recognition sequence for subsequent subcloning into the pKC3 vector. Similarly oligonucleotide EG5 that hybridises to the 3' end of the chimeric cDNA contained a Sall recognition site for subsequent subcloning of the chimaeric cDNA into the pKC3 vector. The PCR reaction were conveniently performed under the standard conditions i.e. oligonucleotides and template (quantities as required - see above) were mixed with 2.5U of Taq DNA polymerase in 10mM Tris-HCl pH 8.3, 50mMKCl, 1.5MgCl₂ (Varied according to oligonucleotide primer combination). Twenty-five cycles were performed each cycle consisting of denaturation at 94°C for 1 minute, annealing for 2 minutes at appropriate temperature and extension at 72°C for 3 minutes.

Cloning of hFcεRI cDNA.

1st strand cDNA was produced from human PBL (26). PCR was performed on 1st strand cDNA using oligonucleotide primers MDH13 5'TTAGATCTCAGCACAGTAAGCACC3' which hybridizes to nucleotides position 1 to 17 of FcεRI (non-coding strand) (12) and MDH14-5' TTTAGATCTAAATTGAACATCTCTTTTAC 3' (positions 1042 TO 1062). Both oligonucleotides contain a Bgl-II sit at their 5' ends for subcloning of the PCR product into pKC3 vector. The nucleotide sequence of the cloned FcRI DNA was determined by dideoxy-nucleotide sequencing (13,14) and is shown in Figure 7.

FcγR cDNA.

Mouse FcγRII and FcγRI cDNA used herein have been previously described (4, 5, 7). The human FcγRIIa cDNA sequence used herein has been described (6, Australian Patent Serial No. 595623) and the sequence shown in Figure 6.

Production of Monoclonal Antibodies. BALB/c mice, 8-10 week females, were immunised by weekly intraperitoneal injection of whole cells (0.5mls of 10⁸-10⁹ cells/ml) for a least 3 weeks with either the K562 cell line or L-cells transfected with the human FcγRII 3.0 cDNA (Tf3.0) (6). Cell fusions using the NS-1 myeloma cell line were performed as described (29). Hybridoma supernatants were screened for MAb by two stage rosetting of target cells using sheep anti-mouse immunoglobulin coupled to SRBC via CrC12 (28). Target cells used for screening were both K562 and Tf3.0 cell line. Positive hybridmas were then cloned by limiting dilution at least

twice and then grown as ascites tumors in (CBAxBALB/c)F1 mice primed with pristane. MAbs were purified from ascites using Protein A Sepharose chromatography. Other MAb used in the study included IV.3 (IgG2b) and CKM5 (IgG1) both standard anti CD32 MAb (22, 23). Anti-Ly2,1 antibody 49.11.1 (IgG2a) and 49-17.1 (IgG), and 5084-4.1 (IgG2a) anti-Ly-12.1 (IgG2A) used as a negative control antibodies.

Quantitative immunofluorescence.

Binding of MAb to haemopoietic cell lines was quantitated on a Facscan by immunofluorescence. 25ul of ascites or serum (1:400 dilution) or aggregated human IgG in PBS was added to target cells (5×10^6 cells/ml) in PBS-BSA 0.5% and incubated for 45 minutes on ice. The monocyte cell lines U937 and HL-60 were pre-incubated with 25 ul of 5mg/ml of human immunoglobulin to block non specific Fc binding to high affinity Fc γ RI. Cells were washed and resuspended in fluorescein isothiocyanate (FITC) sheep anti-mouse Ig F(ab) 2 (silenus) 1:50 dilution) was then incubated on ice for 30 minutes in the dark. Cells were washed and resuspended in 0.5ml PBS-BSA 0.5%.

Inhibition of EA Rosetting.

The ability of the MAb to block Fc binding was determined by inhibition of rosetting with polyclonal rabbit antibody coated erythrocytes (EA). The inhibition assay was performed as follows: 50ul of the blocking antibody ascites or serum was serially diluted and incubated with 50ul of Fc γ R bearing target cells at a concentration of 5×10^6 /ml for 45 minutes on ice. Cells were washed free of excess antibody. 50ul of EA was added to the cell and spin at 200g for 4 minutes. The EA's and cells were incubated for 30 minutes on ice. Cells were stained with Ethyl Violet and a typical field of 100 cells was assessed for rosette formation (at least 5 RBC bound or 50% of cell surface covered). Target cells used were K562, Daudi and Tf3.0. Sensitisation of sheep erythrocytes with antibody was performed as described (5, 29).

Competitive inhibition Assays.

Purified antibodies (100ug) were labelled with carrier free I^{125} using the Chloramine T method (29) but labelling performed for 30 seconds on ice with a

Chloramine T concentration of 1mg/ml. Free iodine was removed on a Sephadex PD-10 column (Pharmacia). Flexible microtitre plates were coated with 5% skim milk overnight to reduce non specific binding. Serial two-fold dilutions starting at a final concentration of 25ug/ml of unlabelled antibody (25ul) was mixed with 25ul of a fixed predetermined dilution of radiolabelled antibody. Finally 50ul of target cells (10^7 cells/ml) was added to the mixture of labelled and unlabelled antibody, and incubated at 4°C for 4 hours. Cells were washed and assayed for bound radioactivity. Value of radioactivity bound to cells in absence of unlabelled antibody was taken as 100% binding. The % inhibition was calculated as follows:-

$$\frac{(\text{max. cpm}) - (\text{xcpm})}{\text{max.cpm}} \times 100$$

where x=cpm of assayed cells and max.cpm = 100% binding value. Target cells used where FcγRII cDNA transfected L-cells and K562 cell.

Unlabelled irrelevant antibodies 49-11.1 or 49.17.1 antibodies were used to assess non-specific binding.

Immunoprecipitation.

Cell suspensions (5×10^7 to 10^8) were surface labelled with carrier free I^{125} using a modified lactoperoxidase method (29). I^{125} (10ul of 1mCi/ml; Amersham) and lactoperoxidase (80ul, 1mg/ml PBS; Sigma) was added to the cell suspension and surface radioiodination initiated by sequential addition of H_2O_2 (BDH) (20ul) of 1:27,000, 1:900, 1:2000, 1:1000 dilution of 30% v/v solution in PBS) at 2 minute intervals. The reaction was stopped by addition of cold PBS and the cells washed twice in PBS. Cells were then solubilised in lysis buffer (0.01M Tris.Ha), 15M NaCl, 0.5% NP 40, 1mM EDTA and 1mM Phenyl sulphonyl fluoride pH=8) at 4°C for 2 hours. Nuclei were removed by centrifugation at 10,000g for 10 min. Free iodine was removed on a Sephadex PD-10 column. Immunoprecipitation was carried out by incubating 50ul antibody (Fab/Fab²) conjugated sepharose beads (washed in lysis buffer) with 1ml of cell lysate at 4°C overnight. Polyclonal antibody or HAGG were incubated with 50ul of protein A conjugated Sepharose and then with 1ml of cell lysate at 4°C. The following day beads were washed in lysis mix, dissolved in SDS-PAGE sample buffer and analysed on a 10% SDS-PAGE gel under reducing

conditions (29). Dried gels were autoradiographed.

Assay for Human Soluble FcγRII Using a Two Antibody Sandwich Radioimmunoassay.

Two monoclonal antibodies (8.2 and 8.26) to human Fc RII detecting different epitopes were purified from ascities using Protein A Sepharose chromatography (29). Flexible 96 well PVC microtitre plates (Costa) are coated with the first antibody 8.26 using 50–70ul per well at a concentration of 10ug/ml of antibody diluted in 0.005M carbonate/bicarbonate coating buffer pH=9.6 and incubated for 2 hours at 37°C. The plates are then washed three times by flooding the plates in PBS/0.05% Tween 20. The remaining sites in the wells are then blocked by coating the wells with 200 ul of 5% BSA and incubating for 1 hour at 37°C to reduce non-specific binding. The plate is then washed three times in PBS/0.05% Tween 20. Serum samples are diluted 1:4 in 2% BSA and 50ul is added to each well and incubated at 4°C overnight. The plates are then washed three times in PBS/0.05% Tween 20. The second radiolabelled antibody (8.7) was then added. 50ul of ¹²⁵I labelled 8.7 (50x10⁴ cpm/50ul) is added to each well and incubated for 4 hours at 4°C. The plates were washed four times by flooding in PBS/0.05% Tween 20 and individual wells were cut and counted in Gamma counter.

Transfections.

Transfection of plasmid DNA into COS–7 cells was performed using DEAE dextran as described (10, 13, 14).

Monoclonal antibodies.

The monoclonal anti-FcγRII antibody IV.3 was obtained from Dr Clark Anderson (Ohio State university) and the CIKM5 antibody from Mr Glen Pilkington (Cancer Institute, Melbourne).

The IgG anti-TNP antibodies used have previously been detailed (5). The IgE anti-TNP antibody, TIB–142 was obtained from the ATCC (Maryland, USA). The monoclonal anti-glycophorin antibody was obtained from Dr Leonie Ashman (University of Adelaide).

The 2.4G2 antibody has been described (30).

Fab'₂ or Fab fragments of antibodies were produced as described (29).

Polyclonal anti-FcγRII antibodies.

Rabbit antibody detecting human FcγRII was produced by immunisation of New Zealand White rabbits with purified FcγRII fusion protein. The rabbit were immunized at regular intervals five times 3–5mg of fusion protein. The first immunisation was performed intradermally in multiple sites in complete Freund's adjuvant and subsequent immunisations in incomplete Freund's adjuvant.

The bacterial fusion protein was produced using the pATH21 vector (31) by cloning the large Pst-1 – XbaI fragment of FcγRIIa cDNA (Figure 6) into the PstI site and XbaI sites of pATH21. The XbaI site being introduced by PCR mutagenesis into the FcγRLLa cDNA at position 627–632.

The induction and purification of the fusion protein by electrophoresis was performed as described (31)

Scatchard Analysis.

Scatchard analysis was performed as described (32) using monomeric mouse I¹²⁵ – IgG2a prepared by incubating 100ug of IgG2a (in 50ul of 0.3M phosphate buffered saline pH7.4) with 500uCi Na¹²⁵ (Amersham UK) in the presence of chloramine – T Merck) at 1mg/ml in PBS pH7.4, for 30 sec. on ice. Labelling reactions were stopped with 50ul of 2.4mg/ml sodium metabisulphite and 10mg/ml tyrosine; free ¹²⁵ was removed from labelled protein by passage through a PD-10 Sephadex G-25 column (Pharmacia, Uppsala, Sweden. COS cells transiently transfected with FcR cDNAs were harvested 48hr after transfection, washed twice in PBS containing 0.5% BSA and resuspended to 2x10⁶/ml in L15–0.05% BSA for use in Scatchard analysis. 50ul aliquots of cells were incubated with 50ul serial dilutions of ¹²⁵ – IgG2a in L15 medium for 60 min at 25°C with periodic agitation. Cells were then pelleted by centrifugation through a 3:2 (v/v) mixture of dibutylphthalate and dioctylphthalate oils (Fluka Chemika, Switzerland) and cell bound ¹²⁵ – IgG2a assayed. Parallel experiments were performed under identical conditions in the presence of 100 fold excess of unlabelled IgG2a to correct for non-specific Ig binding.

Oligonucleotides

Oligonucleotides were synthesised as described (27) and are listed in Table 9.

The invention will be further described with reference to the following figures:

Figure 1

Mutagenesis of FcγRI. PCR was employed to introduce an Apa I site between D2 and D3 of FcγRI cDNA to facilitate domain exchange with FcγRII cDNA. Two PCR reactions were performed, one to produce a fragment containing D1 and D2 coding regions and a second PCR to produce a fragment containing D3, the transmembrane and cytoplasmic tail coding regions. Each of the fragments were engineered to have an Apa I site introduced into the FcγRI sequence between the D2 and D3 coding regions – through the use of overlapping and partly complementary oligonucleotide PCR primers (the Apa I site is indicated by solid circles). Each PCR reaction also employed an oligonucleotide primer containing a Sal I site (solid box), thus producing FcγRI DNA fragments containing Sal I and Apa I sticky ends suitable for construction of the chimeric receptor cDNAs (see Fig. 2).

PCR conditions: The complementary oligonucleotide designed to introduce the Apa I site had the following sequences:

MDH1 5' GGTGAA GGGCCC TTTCACCGTGATGG 3'

MDH2 GGTGAAAGGGCCC TTCACCACGCCAG 3'

MDH1 corresponds to nucleotides 573 to 599 and MDH2 corresponds to nucleotide 580 to 605 of the FcγRI cDNA sequence.

Boxed sequence contains the Apa I site.

The oligonucleotides containing the Sal I site were as follows:

MDH3 5' TTT GTCGAC ATGATTCTTACCAGCCTTTGGAGATG 3'

MDH4 5' TTT GTCGAC CCCCGGGGATCCTCTAGAGTCGAC 3'

The boxed sequence contains the Sal I site. MDH3 hybridizes to FcγRI 5' untranslated sequence from position 1–26 the first 8 bases of MDH3 contain 3 spacer nucleotides and 5 bases of the Sal I sequence.

MDH4 hybridizes to the pGEXII vector sequence flanking the 3' end of FcγRI cDNA insert.

The two PCR employed the oligonucleotide pairs MDH1 and MDH3 to produce the D1 and D2 fragment and MDH2 with MDH4 to produce the D3, tm and cytoplasmic tail coding fragment. PCRs were performed under the following

conditions: 1ng of FcγRI cDNA (cloned in the vector pGEXII) was used as a template for amplification of the two fragments outlined above. 25 cycles were performed using a Perkin-Elmer-Cetus DNA thermal cycle in the presence of 500ng of each oligonucleotide primer, 10mM Tris-C1 ph 8.3, 50mM KCl, 1.5mM MgCl₂ and 2.5 units of taq polymerase (Amplitaq - Perkin - Elmer - Cetus). The PCR products were extracted with phenol: chloroform and ethanol precipitated. The precipitates were dried, dissolved in buffer and digested with Sal I and Apa I, extracted, precipitated and used in subsequent cloning steps see Fig. 2.

Figure 2

Construction of expressable chimeric FcR cDNA's.

Chimeric cDNA's were generated by replacing FcγRII coding domains in the expression vectors with FcγRI coding domains generated by PCR. (A) FcγRII cDNA was subcloned into the Pst I site of the expression vectors pKC3 and pKC4 (derived from the parental vector pKO-neo Van Doren, et al J. Virol. 50 (1984) in expressable orientation [pKC3 and pKC4 differ only in the orientation of the polylinker polycloning sites such that the Sal I site flanks the 5' or 3' end of the cDNA insert in pKC3 of pKC4 respectively].

(B) To produce the FcγRI-II chimera (consisting of D1 and D2 of FcγRI linked to the transmembrane and cytoplasmic coding regions of FcγRII) D1 and D2 of FcγRII were removed from pKC3-FcγRII by digestion with Sal I and Apa I and (C) replaced with a Sal I Apa I fragment containing D1 and D2 of FcγRI derived by PCR (see figure 1).

(D) Similarly, for generation of the FcγRII-I chimera (consisting of d1 and d2 of FcγRII linked to D3, the transmembrane and cytoplasmic coding domains of FcγRI) the cDNA sequences encoding FcγRII transmembrane and cytoplasmic regions were removed by digestion with Sal I and Apa I, and (E) replaced with Sal I - Apa I fragment containing D3, the transmembrane and cytoplasmic coding domains of FcγRI derived by PCR (see Figure 1).

Figure 3

Nucleotide and amino acid sequence of chimeric Fc receptors. Nucleotide positions are numbered in decades below the line in a 5' to 3' direction. Untranslated

sequence is shown in closed type.

A. Chimeric Fc γ RI/II. Fc RI derived cDNA sequence from positions 9 to 594, Fc γ RII from 595 to 1244.

B. Chimeric Fc γ RII/I. Fc γ RII derived cDNA sequence from positions 1 to 662, Fc γ RI from 663 to 1348 between Fc γ RI and Fc γ RII domains. The Apa I restriction, site GGGCCC in both chimeric sequences is underlined. Sal I sites introduced into Fc γ RI sequence by PCR to facilitate cloning are boxed.

Figure 4

IgG immune complex binding to chimaeric Fc γ R using antibody sensitized erythrocytes (EA). COS-7 cells were transfected with cDNA encoding Fc γ RI (A,F,G); Fc γ RII (D); (Fc γ RI/II (C,I,J); Fc γ RII/I (B); vector only (E,H). The transfected COS cell monolayers were tested for Fc binding using erythrocytes sensitised with antibody. Rabbit IgG-EA were used in (A-E) and mouse IgG1-EA (G,H,J) and mouse IgG2a-EA (F,I). Methods described in Materials and Methods Section.

Figure 5

Scatchard analysis of IgG2a binding to Fc γ RI transfected COS-7 cells. Data has been subjected to linear regression analysis.

Figure 6

Nucleotide and deduced amino acid sequence of human Fc γ RIIa (6). Amino acid positions are numbered above the line in decades commencing at the proposed amino acid terminal end. (Signal sequence is numbered from residue -34 to -1). Nucleotide positions are numbered at the end of the line. Oligonucleotide primers used in the PCR reactions for construction of the chimaeric cDNA are positioned at their priming sites with 5' to 3' direction indicated by half arrow heads. Solid lines represent oligonucleotide sequence derived from the Fc γ RII cDNA template, dotted lines represent oligonucleotide sequence derived from Fc ϵ RI cDNA template (Figure 7). Solid boxes represent 5' terminal EcoRI sites, solid circles 5' terminal Sall sites.

Figure 7

Nucleotide and deduced amino acid sequence of human Fc ϵ RI. Amino acid positions are numbered above the line in decades commencing at the proposed amino

acid terminal end (signal sequence is numbered from residue -25 to -1). Nucleotide positions are numbered at the end of the line. Oligonucleotide primers used in the PCR reactions for constructions of the chimaeric cDNA are positioned at their priming site with 5' to 3' direction indicated by half arrow heads. Dotted lines represent oligonucleotides sequence derived from the FcεRI cDNA template, solid line represents oligonucleotide sequence derived from FcγRII cDNA template (Figure 6). Solid boxes represent 5' terminal EcoRI sites.

Figure 8

Rosetting of COS-7 cells transfected with FcR cDNA. COS 7 cell monolayers were transfected with human FcγRII cDNA (A,B); Chimaera 1 cDNA (C,D); Chimaera 2(E,F); Chimaera 3 (G,H); chimaera 4 (I,J). Seventy-two hours later the binding of antibody sensitised erythrocytes was assessed using IgE-EA (A,C,E,G,I) or IgG-EA (B,D,F,H,J).

Figure 9

Fluorescence histograms of immunofluorescence of anti-FcγRII monoclonal antibody staining of Daudi cells of U937 cells as indicated. Horizontal axis represents fluorescence intensity (log scale) the vertical axis represents relative cell number.

Figure 10

Competitive binding of unlabelled monoclonal anti-Fc RII antibodies. Competitive assay performed using L-cells transfected with human FcγRII cDNA HFc 3.0 Radiolabelled antibodies are (A), IV.3; (B), CIKM5; (C), 8.2; (D), 7.30. The cold competitors are shown, Ly-2.1 (49-11.1); IV.3; CIKM5; 8.2; 7.30.

Figure 11

Immunoprecipitation of FcγRII from surface labelled K562,(A) or Daudi,(B) cells. Molecular weight standards (Mrx10, (Std) are indicated on the left side of the figure. Antibodies (Fab or Fab'2 fragments) used are IV.3, CIKM5, 8.2, 8.7, 8.26. Human aggregated IgG (HAGG) intact Ly-12.1; 1705(5084-4.1). The negative control for the polyclonal antibody was immunoprecipitation from FcγRII-CEM cells.

Figure 12

Detection of circulating FcγRII in the serum of normal or diseased individuals. (A) mean cpm \pm 1 S.E. of bound anti-Fc RII in the patient groups indicated. P values are calculated for comparison of patient groups and normals. The numbers (n) of individuals tested within each patient group are indicated. (B) Dot plot of data obtained for individual patients within each group. Each point represents number of cpm (labelled anti-Fc RII antibody) bound.

The entire contents of the provisional specification lodged with Australian Patent Application of which this is the complete specification is hereby imported into this specification and forms part of the disclosure of this specification. The claims form part of the disclosure of this specification.

DEFINITIONS:**Extra functional region:-**

Part of a molecule, having function other than immunoglobulin binding.

Functional region:-

Part of molecule capable of interacting with immunoglobulins or their fragments whether the immunoglobulins or fragments are monomeric in nature, aggregated or immune complexes.

Immunoglobulin binding molecule:-

Any molecule capable of interacting with immunoglobulins or their fragments whether the immunoglobulins or fragments are monomeric in nature, aggregated or immune complexes.

Functional equivalents thereof:-

Variants on the materials the subject of this specification are possible:-

A. Sequence Variation

The nucleotide sequences encoding the receptor can be variable:-

1. Because of the degeneracy of the genetic code nucleotide change does not necessarily bring about a change in the amino acid encoded.
2. Two or three nucleotide changes can give rise to the same amino acid.
3. Changing one or two nucleotides may result in a conservative amino acid change unlikely to greatly affect the function of the protein.
4. Allelic variations. Variations in nucleotide sequence and resultant amino acid sequences of the encoded protein may occur between individual members of the same species. These variations arise from changes in the nucleotide sequences encoding the protein. Thus different forms of the same gene (called alleles) give rise to protein of slightly different amino acid sequence but still have the same function.
5. Proteins having the same function, e.g. immunoglobulin binding, may arise from related genes. Many protein gene families have been described.
6. Variation may be intentionally introduced by:-
 - (a) Mutating cloned cDNA or genomic DNA by point mutation, rearrangement or insertion of related or unrelated DNA into the cDNA or genomic clones

encoding the functional protein. Such mutated (variant) clones can be used to generate variant proteins or peptides.

- (b) By enzymatic cleavage of the protein (from either in vitro synthesis or normal cell synthesised protein) with or without repair/rearrangement of the cleavage products.
- (c) By chemical modification.
- (d) By irradiation.

Greek symbols:-

α alpha

γ gamma

δ delta

ϵ epsilon

μ mu

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TABLE 1

Binding of IgG subclasses to chimeric receptors

Transfecting DNA	Binding of IgG*					
	Mouse IgG subclasses ^t					
	<u>Human</u> IgG**	<u>Rabbit</u> IgG ^t	G1	G2a	G2b	G3
FcRI	+	+	-	+	-	-
FcRII	+	+	+	+	+	+
FcRI-II	+	+	+	+	+	+
FcRII-I	+	+	+	+	+	+
NIL	-	-	-	-	-	-

* Ig binding assessed by EA rosetting for rabbit and mouse IgG. And

^t Ig binding level where >10 IgG coated erythrocytes bound per adherent Cos cell.

** binding assessed by immunofluorescence using heat aggregated-human poly clonal IgG and quantified by FACS analysis.

Erythrocyte - antibody complexes were prepared as follows:

- (i) Rabbit IgG sensitised erythrocytes were prepared as in Figure 1.
- (ii) EA coated with specific mouse IgG subclasses were produced by using anti-TNP isotype specific monoclonal antibodies (of the IgG1, IgG2a, IgG2b subclasses). Sheep red blood cells were sensitised with TNP by incubating a 10% SRBC suspension with 7 volumes of TNP in PBS (Phosphate buffered saline) for 20 minutes at room

temperature. Sensitised cells were then washed twice in PBS+0.5% BSA and resuspended to a 2% suspension upon which an equal volume of antibody was added and incubated for 60 minutes at room temperature.

- (iii) Human red blood cells directly sensitised with IgG1 or IgG3 anti-glycophorin monoclonal antibodies as described above. EA's were used in rosetting assay again as outlined in Figure 4 legend.

Assays were performed directly in culture dishes on COS-7 cells transiently transfected with FcR expression constructs (Fc RI in the pGEXII vector (Ref), Fc RII in pKC3, Fc RII-I in pKC4, Fc RII in pKC3), as described. EA's were then washed twice in PBS-0.5% BSA again resuspended to a 2% suspension for use in rosetting following an approach outlined in Figure 3 legend.

Immunofluorescence was performed on transiently transfected cells. Polyclonal human IgG (5 mg/ml) was aggregated at 65°C for 30 minutes. Binding and immunofluorescence studies were performed as described (29).

TABLE 2

Detection 2.4G2 epitope on chimeric receptors
by blocking of EA rosetting

Transfecting DNA	% Blocking EA rosetting*
FcRI	0
FcRII	80
FcRI-II	80
FcRII-I	80
Vector only **	0

* % blocking relative to transfected cells not treated with 2.4G2 antibody.

** Cos cells were transfected with pKC4 vector DNA.

Blocking assay were performed on Cos 7 cells transiently transfected with FcR expression constructs (see Table 1). Transfected cells in culture dishes were incubated whilst adhered with Fab fragments of the anti-Fc RII monoclonal antibody 2.4G2 (Unkeless; J.C. Exp. Med 150: 580-596, 1979) for 60' at 4°C. Cells (in dishes) were then washed x2 with L-15-0.5% BSA medium, and rabbit EA added to dishes. Rosette formation was assessed as outlined to Figure 3 legend.

TABLE 3 FcR cDNA AND CHIMAERIC FcR cDNA IN THIS STUDY

CHIMAERA NAME	ORIGIN OF DOMAIN*		RECEPTOR COMPOSITION**		ORIGIN OF AMINO ACID RESIDUES*†
	D1	D2	TM/CT	D1 D2	
FcεRI	ε	ε	ε		
FcγRII	γ	γ	γ		
1	ε	ε	γ		[ε(-25 → 169), γ (170 → 281)]
2	γ	ε	γ		[γ(-34 → 86), ε (87 → 169), γ(170 → 281)]
3	ε	γ	γ		[ε(-25 → 86), γ (87 → 281)]
4	ε	ε/γ	γ		[ε(-25 → 128), γ (129 → 281)]
5	ε	γ/ε	γ		[ε(-25 → 86), γ (87 → 128), ε(129 → 169), γ(170-281)]
6	γ	ε/γ	γ		[γ(-34 → 86), ε(87 → 128), γ(129 → 281)]
7	γ	γ/ε	γ		[γ(34 → 128), ε (129 → 169), γ (170 → 281)]

* D1, EC domain 1; D2, EC domain 2; TM, transmembrane; CT, cytoplasmic tail, ε, derived from FcεRI; γ, derived from FcγRII. Diagrammatic representation of origin of the regions of the Fc receptors. Unshaded regions derived from FcεRI; shaded regions derived from FcγRII. Origin of leader sequences not shown. The nucleotide and amino acid sequence of all native and chimaeric FcR was determined by dideoxy nucleotide sequence analysis (12, 13).

† Amino acid residues of the receptors. Numbering indicates the amino acids derived from FcεRI (ε) or FcγRII (γ) and numbers correspond to amino acid residues in Figures 6 and 7.

TABLE 4 CONSTRUCTION OF CHIMAERIC cDNAs

CHIMAERA NAME	ORIGIN OF DOMAINS			OLIGONUCLEOTIDE Pair I (Template)*	OLIGONUCLEOTIDE Pair II (Template)*
	D1	D2	TM/CT		
FcγRII	γ	γ	γ	-	-
FcεRI	ε	ε	ε	-	-
1	ε	ε	γ	<u>EG6</u> + EG1 (FcεRI)	EG2 + <u>EG5</u> (FcγRII)
2	γ	ε	γ	<u>NR1</u> + EG11 (FcγRII)	EG10 + <u>EG5</u> (Chimaera 1)
3	ε	γ	γ	<u>EG6</u> + EG9 (FcεRI)	EG8 + <u>EG5</u> (FcγRII)
4	ε	ε/γ	γ	<u>EG6</u> + EG14 (FcεRI)	EG15 + <u>EG5</u> (FcγRII)
5	ε	γ/ε	γ	<u>EG6</u> + EG12 (Chimaera 3)	EG13 + <u>EG5</u> (Chimaera 1)
6	γ	ε/γ	γ	<u>NR1</u> + EG14 (Chimaera 2)	EG15 + <u>EG5</u> (FcγRII)
7	γ	γ/ε	γ	<u>NR1</u> + EG12 (FcγRII)	EG13 + <u>EG5</u> (Chimaera 1)

* Underlined oligonucleotides used in SOE reaction; templates for PCR reactions shown in parentheses.

TABLE 5 PROPERTIES OF NATIVE FcγRII, FcεRI AND CHIMAERIC FcR

CHIMAERIC NAME	ORIGIN OF DOMAIN*		IMMUNE COMPLEX BINDING**							ANTI FcγRII MoAb BINDING†		
	D1	D2	TM/CT	IgG	IgE	8.7	7.30	8.2	8.26	IV-3	CIKM5	
FcεRI	ε	ε	ε	-	-	-	-	-	-	-	-	
FcγRII	γ	γ	γ	+	-	+	+	+	+	+	+	
1	ε	ε	γ	-	+	-	-	-	-	-	-	
2	γ	ε	ε	-	+	-	-	-	-	-	-	
3	ε	γ	γ	+	-	+	+	-	-	+	-	
4	ε	ε/γ	γ	+	+	ND	ND	ND	ND	ND	ND	
5	ε	ε/γ	γ	-	-	ND	ND	ND	ND	ND	ND	
6	γ	ε/γ	γ	+	+	ND	ND	ND	ND	ND	ND	
7	γ	γ/ε	γ	-	-	ND	ND	ND	ND	ND	ND	

* D1, EC domain 1; D2, EC domain 2; TM, transmembrane; CT, cytoplasmic tail; ε, derived from FcεRI; γ, derived from FcγRII.

** immune complex binding assessed by EA rosetting using IgG and IgE sensitised erythrocytes. + = formation of rosettes; - = no rosette formation.

† Binding of monoclonal anti FcγR antibodies (MoAb) to cells transfected with native or chimaeric Fc receptors. Binding assessed by immunofluorescence and visualisation by fluorescence microscopy. +, fluorescent staining; -, no staining; ND, not determined.

TABLE 6 CHARACTERISATION AND TISSUE DISTRIBUTION OF MONOCLONAL ANTIBODIES

MoAb	Immunogen	Ig Class	Target Cells												
			Tf3.0	L-cells	K562	DAUDI	RAJI	EBV	U937	ThP1	HL-60	CEM	JURKATT	MOLT4	PEER
			B cells												
IV.3	K562	$\gamma 2b$	+++	-	+++	+	-	+	+++	+++	+	-	-	-	
	CIKM5	K562	+++	-	+++	+	+	+	+++	++	+	-	-	-	
8.2	K562	$\gamma 1$	+++	-	+++	+	-	+	+++	+++	+	-	-	-	
	K562	$\gamma 1$	+++	-	+++	+++	+	+	+++	+++	+	-	-	-	
8.26	K562	$\gamma 2b$	+++	-	+++	++	+	+	+++	+++	+	-	-	-	
	Tf3.0	$\gamma 1$	+++	-	+++	+++	+	+	+++	+++	+	-	-	-	

* % reactivity with haemopoietic cell lines and human Fc γ RII transfected L cells compared to the negative control mAb 49.11.1. Ascites or serum was used at dilution of 1:400 0-10% reactivity = Negative (-); 10-50% reactivity = +; 50-75% reactivity = ++; 75-100% reactivity = ++++. The control mAb 49.11.1 was negative in all cases.

Table 7

INHIBITION OF IMMUNE COMPLEX BINDING (EA-ROSETTING)

mAb	Target Cell		
	K562	Tf3.0	DAUDI
IV.3	100*	100	100
CIKM5	44	0	0
8.2	100	100	100
8.7	78	88	100
8.26	100	100	100
7.30	44	75	100
49.11.1	0	0	0

* Percentage of inhibition of EA rosette formation using all indicated antibodies at a 1:2000 final concentration of ascites or serum. Target cells indicated.

TABLE 8

COMPETITIVE BINDING ASSAY*

Cold Competitor	RADIOLABELLED ANTIBODY					
	IV.3	8.2	8.26	CIKM5	8.7	7.30
IV.3	+	+	+	+	+	+
8.2	+	+	+	+	-	-
8.26	+	+	+	+	-	-
CIKM5	±	±	±	+	±	-
8.7	±	-	-	±	+	+
7.30	±	-	-	±	+	+

* K562 is the target cell

+; % inhibition equivalent to that obtained with the same unlabelled antibody

e.g. unlabelled IV.3 inhibition of labelled IV.3.

±; partial or minimal inhibition of radiolabelled antibody

-; no inhibition of radiolabelled antibody.

TABLE 9 OLIGONUCLEOTIDES USED IN THE GENERATION OF CHIMAERIC FcR

NAME:	SEQUENCE 5'-3'	NUCLEOTIDE POSITION ON FcεRI/FcγRII cDNA** (5' to 3' of oligonucleotide)
EG1	5 GGCACCTTGACAGTAATGTTGAGGG3	FcγRII 628-621; FcεRI 621-604.
EG2	5 CATTACTGTACAAGTGCCAGCATG3	FcεRI 612-621; FcγRII 621 to 635.
EG5†	5 TTTGTCGACCACATGGCATAACG3	FcγRII 981-967
EG6†	5 TTTGAATTCAGCACAGTAAGCACC3	FcεRI 6-22
EG8	5 TTCAGTGACTGGCTGGTGCTCCAG3	FcεRI 364-372; FcγRII 372-386
EG9	5 ACCAGCCAGTCACTGAAGACTTCC3	FcγRII 379-372; FcεRI 372-357
EG10	5 CTTTCCGAATGGCTGCTCCTTCAG3	FcγRII 363-371; FcεRI 373-387
EG11	5 AGCAGCCATTCCGGAAGCACAGTC3	FcεRI 380-373; FcγRII 371-356
EG12	5 TACCAGTATTTCTGGGATTTTCCATT3	FcεRI 506-499; FcγRII 497-479
EG13	5 TOCCAGAAATACTGGTATGAGAAOCAC3	FcγRII 489-497; FcεRI 499-516
EG14	5 CGGGAGAACTTGAGAGCTTCAOCATC3	FcγRII 505-498; FcεRI 498-481
EG15	5 CTCTCAAGTTCTCCCGTTTGGATCC3	FcεRI 491-498; FcγRII 498-514
NR1†	5 TACGAATTCCTATGGAGACCCAAATGTC3	FcγRII 10-30

* Underlined oligonucleotides complementary to coding strand

** Sequence numbers taken from Figures 6, 7.

† These oligodeoxynucleotides also contain non homologous sequence including restriction enzyme recognition sequences.

CLAIMS:

1. A hybrid immunoglobulin binding molecule capable of binding to any one class or a plurality of classes of antibody molecules.
2. A hybrid immunoglobulin binding molecule according to claim 1 which is derived from any Fc receptor (FcR).
3. A hybrid Fc receptor molecule according to claim 2 comprising one or more functional regions of a first FcR linked to one or more functional regions of a second FcR or consecutive FcR.
4. A hybrid Fc receptor molecule comprising a polymer or a concatomer of heterogenous or homogenous functional regions.
5. A hybrid FcR according to any one of claims 1 to 4 wherein said functional regions are derived from different FcR molecules.
6. A hybrid FcR according to any one of claims 1 to 5 wherein said functional regions are derived from bacterial, mammalian or other origins.
7. A hybrid FcR according to any one of claims 1 to 6 wherein said functional regions are derived from human, mouse or a combination of human and mouse origins.
8. A hybrid FcR according to any one of claims 1 to 7 which is capable of binding any one of, or a plurality of antibodies selected from the classes IgG, IgM, IgA, IgD and IgE.
9. A hybrid FcR according to any one of claims 1 to 8 wherein said functional regions are derived from any one of FcR, FcγR, FcγR, FcαR, FcεR, FcμR or IgE binding proteins.
10. A hybrid FcR according to any one of claims 1 to 9 wherein the said functional regions are derived from FcγR or FcεR being any one or a combination of the extracellular domains, or parts thereof, of FcγRI, FcγRII, FcγRIII, FcεRI, FcεRII or homologues or functional parts thereof.
11. A hybrid FcR according to any one of claims 1 to 10 wherein said functional domains comprise one or more extracellular domains, or parts thereof, derived from FcγRII of mammalian origin in combination with any one extracellular domain derived from the following:-

- 51 -

mammalian FcγR
mammalian FcεRI,
mammalian FcεRII,
mammalian FcγRI,
mammalian FcγRIII,
mammalian FcαR,
mammalian FcμR, or
mammalian IgE binding proteins

12. A hybrid FcR according to any one of claims 1 to 11 wherein said functional regions comprise the extracellular domain or parts thereof derived from FcγRI being amino acids 1-171 linked to the transmembrane region and cytoplasmic tail of FcγRII being amino acids.

13. A hybrid FcR according to any one of claims 1 to 11 wherein said functional regions comprise extracellular domains or parts thereof, derived from FcγRII being amino acids 1-174 linked to the third extracellular domain, transmembrane region and cytoplasmic tail of FcγRI being amino acids 174-380.

14. A hybrid FcR according to any one of claims 1 to 11 wherein said functional region comprise an extracellular domain, or parts thereof, having greater than 90% homology to FcγRII or FcεRI of human or mouse origin linked to an extracellular domain of FcεRI of human or mouse origin.

15. A hybrid FcR according to any one of claims 1 to 11 wherein said functional regions comprise an extracellular domain, or parts thereof, having greater than 50% homology to FcγRII or FcεRI of human or mouse origin linked to an extracellular domain of FcεRI of human or mouse origin.

16. A hybrid FcR according to any one of claims 1 to 11 wherein said functional regions comprise an extracellular domain, or parts thereof, having greater than 35% homology to FcγRII or FcεRI of human or mouse origin linked to an extracellular domain of FcεRI of human or mouse origin.

17. A hybrid FcR according to any one of claims 1 to 11 wherein at least one functional region comprises an extracellular domain, or parts thereof, derived from FcγRII having the following amino acid sequence :-

- Phe Ser Arg Leu Asp Pro Thr Phe Ser Tle Pro Gln Ala Asn His Ser His Ser Gly Asp Tyr His Cys Thr Gly Asn Ile Gly Tyr Thr Leu Phe Ser Ser Lys Pro Val Thr Ile Thr Val, or functional equivalent thereof.

18. A hybrid FcR according to any one of claims 1 to 11 wherein at least one functional region comprises an extracellular domain, or parts thereof, derived from FcεRI having the following amino acid sequence : -

- Trp Leu Leu Leu Gln Ala Ser Ala Glu Val Val Met Glu Gly Gln Pro Leu Phe Leu Arg Cys His Gly Trp Arg Asn Trp Asp Val Tyr Lys Val Ile Tyr Tyr Lys Asp Gly Glu Ala Leu Lys, or functional equivalent thereof.

19. A hybrid FcR according to any one of claims 1 to 18 wherein said molecule is not bound to a membrane and is soluble in physiological and or non physiological buffers.

20. A hybrid FcR according to any one of claims 1 to 18 wherein said molecule is linked to any transmembrane region and/or cytoplasmic tail.

21. A hybrid FcR according to claim 20 wherein said transmembrane region and cytoplasmic tail are derived from FcR.

22. A hybrid FcR according to any one of claims 1 to 121 being a chimera comprising a first extracellular domain of FcγRII, a second extracellular domain of FcεRI and a transmembrane region and cytoplasmic tail of FcγRII.

23. A hybrid FcR according to any one of claims 1 to 21 being a chimera comprising a first extracellular domains of FcεRI containing amino acids 1 - 169 of FcεRI linked to amino acids 170-281 of FcγRII.

24. A hybrid FcR according to any one of claims 1 to 21 being a chimera comprising a first extracellular domain of FcεRI being amino acids 1-86 linked to a second extracellular of FcγRII being amino acids 87-169 linked to a transmembrane region and cytoplasmic tail of FcγRII being amino acids 170-281.

25. A hybrid FcR according to any one of claims 1 to 21 being a chimera comprising a first extracellular domain of FcεRI containing amino acids 1-86 linked to part of the second extracellular domain of FcεRI being amino acids 87-128 linked to part of the second extracellular domain of FcγRII being amino acids 129-169

linked to a transmembrane region and cytoplasmic tail of FcγRII being amino acids 170-281.

26. A hybrid FcR according to any one of claims 1 to 21 being a chimera comprising a first extracellular domain of FcεRI being amino acids 1-86 linked to part of the second extracellular domain of FcγRII being amino acids 87-128 linked to part of the second extracellular domain of FεRI being amino acids 129-169 linked to a transmembrane and cytoplasmic tail of FcγRII being amino acids 170-281.

27. A hybrid FcR according to any one of claims 1 to 21 being a chimera comprising a first extracellular domain of FcγRII being amino acids 1-86 linked to part of the second extracellular domain of FcγRII being amino acids 87-128 linked to part of the second extracellular domain of FcεRI being amino acids 129-169 linked to the transmembrane and cytoplasmic tail of FcγRII being amino acids 170-281.

28. A hybrid FcR according to any one of claims 1-21 being a chimera comprising the first extracellular domain of FcγRII being amino acids 1-86 linked to the second extracellular domain of FcεRI being amino acids 87-169 linked to a transmembrane and cytoplasmic tail of FcγRII being amino acids 170-281.

29. A hybrid FcR according to any one of claims 1-21 being a chimera comprising the first extracellular domain of FcγRII being amino acids 1-86 linked to part of the second extracellular domain of FcεRI being amino acids 87-128 linked to part of the second extracellular domain of FcγRII being amino acids 129-169 linked to the transmembrane and cytoplasmic tail of FcγRII being amino acids 170-281.

30. A hybrid FcR according to any one of claims 1 to 29 further comprising one or more extra functional regions of FcR as hereinbefore defined.

31. The amino acid sequence comprising the hybrid FcR according to any one of claims 1 to 31.

32. A nucleotide sequence capable of encoding an amino acid sequence according to claim 31.

33. The nucleotide sequence of claim 32 comprising a cDNA or genomic DNA clone capable of encoding the amino acid sequence according to claim 31.

34. A method of producing hybrid immunoglobulin binding molecules

according to any one of claims 1 to 31 comprising the steps of linking different functional regions of different FcR molecules to construct a hybrid immunoglobulin binding molecule.

35. A method according to claim 34 comprising ligating a first FcR molecule at the transmembrane junction, ligating a second FcR molecule to obtain a suitable functional or non-functional region and linking said components.

36. A polyclonal antibody which reacts with any one of the hybrid FcR molecules as defined in claims 2 to 31, or parts thereof.

37. A monoclonal antibody which reacts with any one of the hybrid FcR molecules as defined in claims 2 to 31, or parts thereof.

38. A monoclonal antibody being of subclass IgG1 designated 8.2 as hereinbefore defined.

39. A monoclonal antibody being of subclass IgG1 designated 8.7 as hereinbefore defined.

40. A monoclonal antibody being of subclass IgG2b designated 8.26 as hereinbefore defined.

41. A monoclonal antibody being of subclass IgG1 designated 7.30 as hereinbefore defined.

42. Antibodies according to any one of claims 36 to 41 which detect a single epitope specific for FcR.

43. A biological product comprising one or more hybrid FcRs according to any one of claims 2 to 31 or antibodies thereto according to any one of claims 36 to 41 for interaction with a hybrid FcR as defined in any one of claims 2 to 31.

44. A biological product according to claim 43 wherein said interaction comprises any one or a combination of binding, detection, linking, inhibiting function.

45. A biological product according to any one of claims 43 or 44 which is capable of being bound to fixed solid support being a tube, dipstick, multiwell plate or other form made from PVC or other material.

46. A biological product according to any one of claims 43 or 44 which is capable of being bound to biologically active materials including cytokines,

hormones, red blood cells, liposomes, dialysis membranes, etc.

47. A biological product according to any one of claims 43 or 46 which is capable of being labelled with any one of labelling enzymes, radioactive isotopes, metal or non-metal particles, fluorescent molecules, free radicals chemiluminescent or bioluminescent molecules.

48. A biological product according to any one of claim 43 to 47 further comprising anti FcR antibodies or fragments thereof.

49. A diagnostic test kit comprising the biological product of any one of claims 43 to 48.

50. An assay for the FcR being an immunoassay utilizing the antibody or biological product of any one of claims 36 to 48.

51. An assay according to claim 50 being a capture tag assay.

52. A capture: tag assay according to claim 51 wherein the FcR is bound, and different epitopes of the said FcR are utilized for further binding and or antigenic functions.

53. A capture: tag assay according to claim 51 wherein the FcR is free.

54. An assay according to any one of claims 50 to 53 comprising the steps of washing away unbound material, adding biological fluid (e.g. serum), incubating for ½-16 hours, washing unbound materials, adding a second labelled anti-human Ig (labelled for example with radioisotope; enzyme particle or fluorochrome), incubating for 1-24 hours, washing and determining the quantity of labelled antibody bound.

55. A capture tag assay according to any one of claims 51 to 53 wherein said assay utilizes hybrid FcR as a standard or competitive inhibitor.

56. A method of regulating antibody production in vivo by binding a hybrid FcR according to any one of claims 2 to 30 to B cells.

57. The use of the method of claim 56 to control autoimmune disease.

58. The use of the method of claim 56 to control allergy reaction by affecting IgE control.

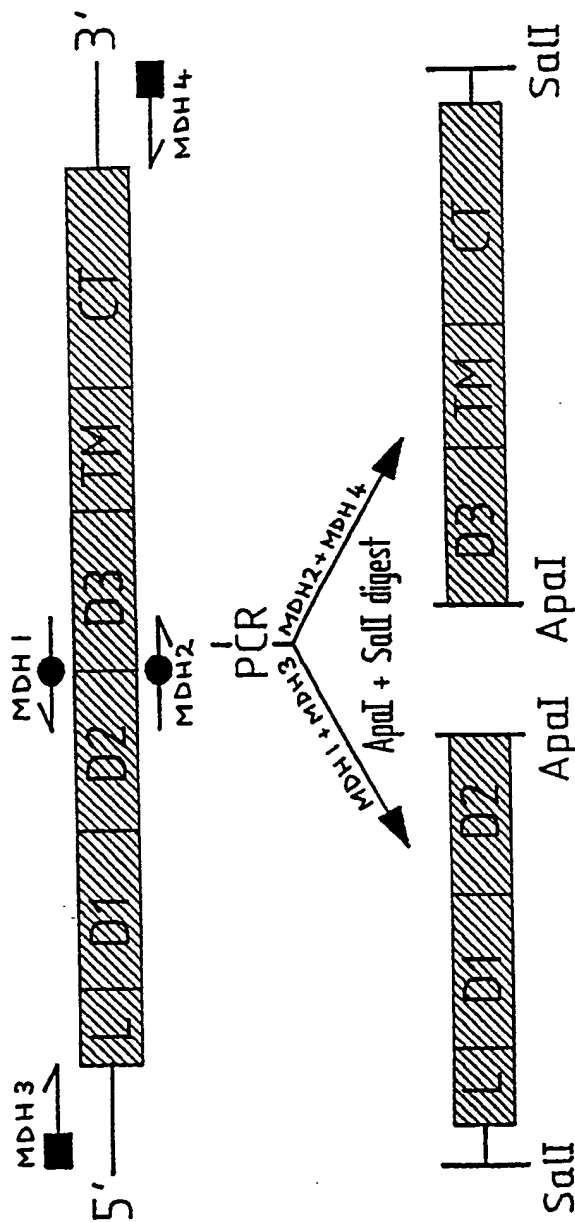
59. A method of regulating allergic reactions by reacting hybrid FcR according to any one of claims 2 to 30 with IgE in vivo.

60. The use of the biological product of any one of claims 43 to 48 for plasmaphoresis to remove immune complexes or pathological antibodies.

61. The use of the biological product of any one of claims 43 to 48 in conjunction with biosensors.

FIGURE ONE

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FIGURE TWO

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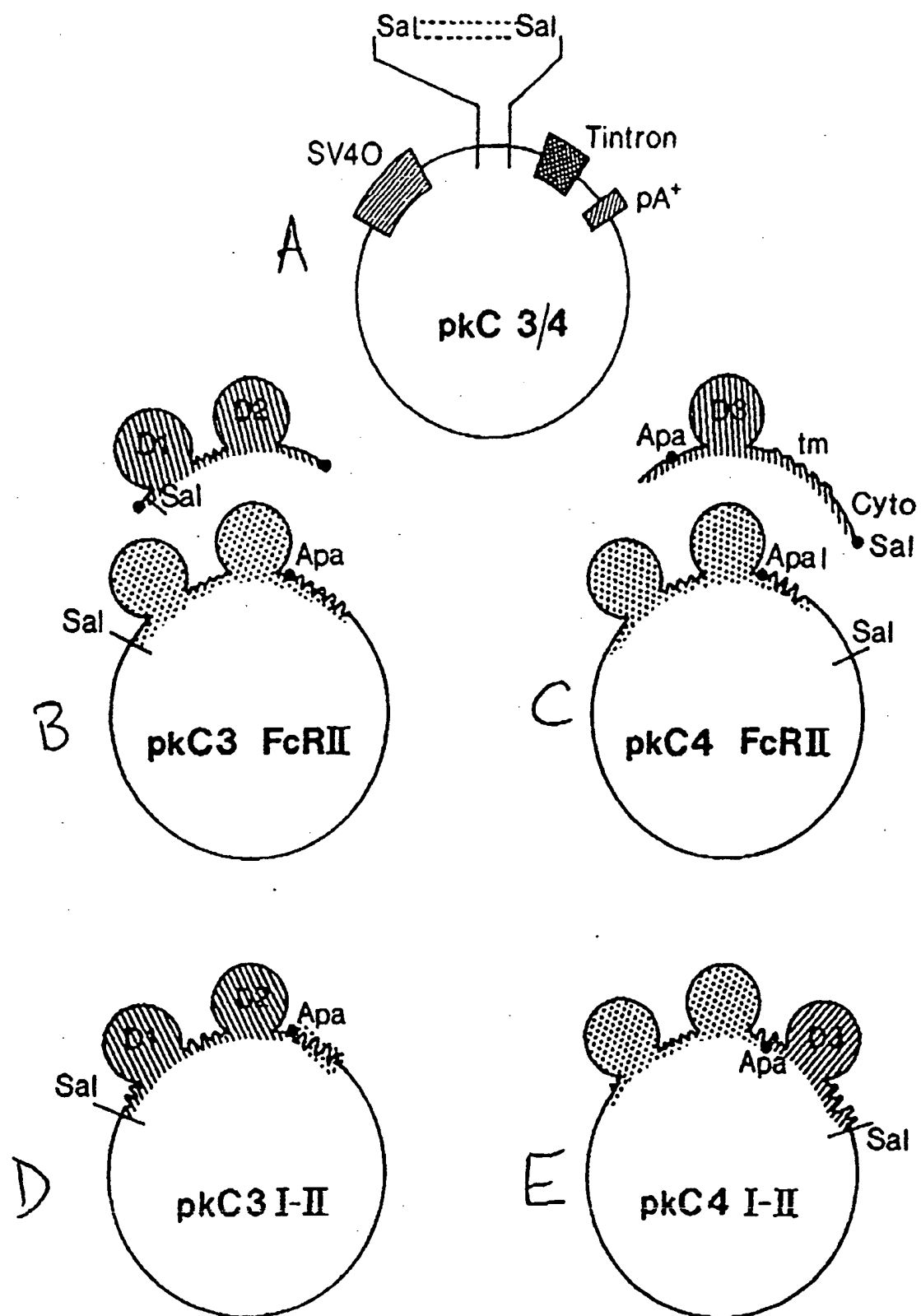


FIGURE THREE A

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Met Ile Leu Thr Ser Phe Gly Asp Asp Met Tip Leu Leu Thr Thr Leu Leu Leu Tip Val Pro Val Gly Gly Val Val
 ATG ATT CTT ACC AGC TTT GGA GAT GAC ATG TGG CTT CTA ACA ACT CTG CTA CTT TGG GTT CCA GTC GGT GGG GAA GTG GTT
 10 20 30 40 50 60 70 80 90

Asn Ala Thr Lys Ala Val Ile Thr Leu Gln Pro Pro Tip Tip Val Ser Ile Phe Gln Lys Glu Asn Val Thr Leu Trp Cys Glu Gly Pro His
 AAT GCC ACC AAG GCT GTG ATC ACC TGG CAG CCT CCA TGG GTC AGT ATT TTC CAG AAG GAA ANT GTC ACT TTA TGG TGT GAG GGG CCT CAC
 100 110 120 130 140 150 160 170 180

Leu Pro Gly Asp Ser Thr Gln Trp Phe Ile Asn Gly Thr Ala Val Gln Ile Ser Thr Pro Ser Tyr Ser Ile Pro Glu Ala Ser Phe
 CTG CCI GGA GAC ACT TCC ACA CAA TGG TTT ATC AAC GGA ACA GCC GTT CAG ATC TCC AGC CCT AGT TAT AGC ATC CCA GAG GCC AGT TTT
 190 200 210 220 230 240 250 260 270

Gln Asp Ser Gly Glu Tyr Arg Cys Gln Ile Gly Ser Ser Met Pro Ser Asp Pro Val Gln Ile His Asn Asp Trp Leu Leu
 CAG GAC ACT GGC GAA TAC AGG TGT CAG ATA GGT TCC TCA ATG CCA ACT GAC CCT GTG CAG TTG CAA ATC CAC AAT GAT TGG CTG CTA CTC
 280 290 300 310 320 330 340 350 360

Gln Ala Ser Arg Arg Val Leu Thr Glu Gly Pro Leu Ala Leu Arg Cys His Gly Trp Lys Asn Lys Leu Val Tyr Asn Val Val Phe
 CAG GCC TCC CGC AGA GTC CTC ACA GAA GGA CCC CTG GCC TTG AGG TGT CAG GGA TGG AAG ANT AAA CTG GTG TAC AAT GTG GTT TTC
 370 380 390 400 410 420 430 440 450

Tyr Arg Asn Gly Lys Ser Phe Gln Phe Ser Ser Asp Ser Glu Val Ala Ile Leu Lys Thr Asn Leu Ser His Ser Gly Ile Tyr His Cys
 TAT AGA ANT GGA AAA TCC TTT CAG TTT TCT TCA GAT TCG GAG GTC GCC ATT CTG AAA ACC AAC CTG AGT CAC AGC GGC ATC TAC CAC TGC
 460 470 480 490 500 510 520 530 540

Ser Gly Thr Gly Arg His Arg Tyr Thr Ser Ala Gly Val Ser Ile Thr Val Lys Gly Pro Lys Ser Arg Ser Leu Pro Val Leu Thr
 TCA GGC ACG GGA AGA CAC CGC TAC ACA TCT GCA GGA GTG TCC ATC ACG GTG AAA GGG CCG MAG TCC ACC AGG TCT TTA CCA GTA TTG ACA
 550 560 570 580 590 600 610 620 630

Ile Val Ala Ala Val Thr Gly Ile Ala Val Ala Ile Val Ile Leu Val Ser Leu Val Tyr Leu Lys Lys Gln Val Val Pro Ala
 ATT GTG GCT GCT GTC ACT GGG ATT GCT GTC GCA GCC ATT GTT ATT ATC CTA GTA TCC TTG GTC TAT CTC AAG AAA AAG CAG GAT CCA GCT
 640 650 660 670 680 690 700 710 720

Leu Pro Gly Asn Pro Asp His Arg Glu Met Gly Glu Thr Leu Pro Glu Glu Val Gly Glu Tyr Arg Gln Pro Ser Gly Gly Ser Val Pro
 CTC CCA GGA AAC CCT GAT CAC AGG GAA ATG GGA GAA ACC CTT CCA GAG GAA GTA GGT GAG TAC AGA CAG CCC TCT GGG GGC TCA GTG CCT
 730 740 750 760 770 780 790 800 810

Val Ser Pro Gly Pro Ser Gly Leu Glu Pro Thr Ser Ser Ser Pro Tyr Asn Pro Asp Leu Glu Glu Ala Pro Lys Thr Glu Ala
 GTC AGC CCA GGG CCT CCA TCT GGA CTG GAG CCA ACA AGC AGC CCA TAC ANT CCT CCT GAT CTG GAA GAA GCT CCC AAA ACT GAG GCT
 820 830 840 850 860 870 880 890 900

Glu Asn Thr Ile Thr Tyr Ser Leu Leu Lys His Pro Glu Ala Leu Asp Glu Thr Glu His Asp Tyr Gln Asn His Ile ***
 GAG AAC ACG ATC ACC TAC TCA CTC CTC AAG CAT CCC GAA GCC TTG GAT GAA GAA ACA GAG CAG CAT GAT TAC CAA AAC CAC ATT TAG TCTCCCT
 910 920 930 940 950 960 970 980 990

TGGCATTGGAAAGCAAGCCAGAAAGCCAGGATCTAGTGTCTCTGGTCCAGGGATGCTGTAGATATTAAAGAAACATCCAGAGTCATCTGTGAGTCCTGAAACCAAGACAC
 1001 1011 1021 1031 1041 1051 1061 1071 1081 1091 1101 1111

TAGGAGATTGGTTCCCAATGGTTGACTGTACTAATGACTCCCATTAAGTTCAGCTTCCCACTCCAGACTCTCTGTATCGATCCACACTGCCACTAAATTAATCACTTCTGCCGT
 1121 1131 1141 1151 1161 1171 1181 1191 1201 1211 1221 1231

TNAGAGACTGCAG
 1241

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FIGURE THREE B

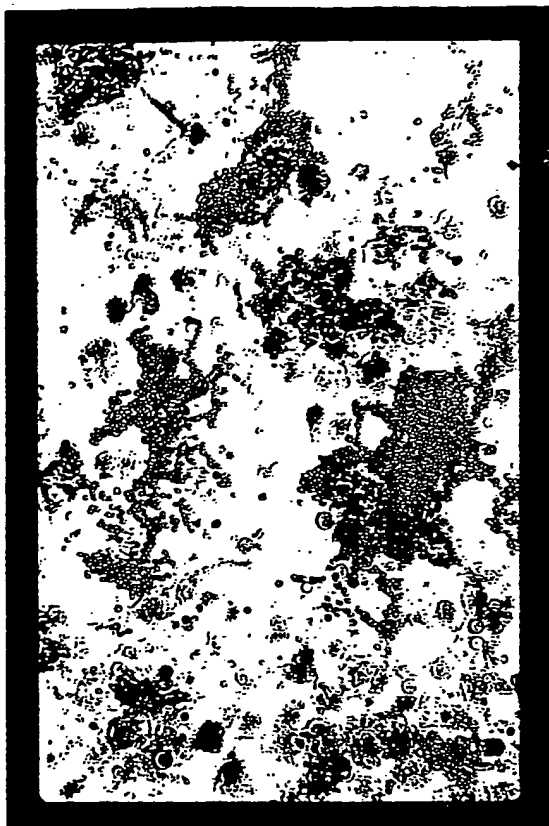
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CTGCAGACTCGCTCCAGAGCTGATGGGAATCTGCGCTTCTACTGATCCCC ATG GAG AAC TGG ACT Thr Val His Val Phe Ser Arg Thr Leu Cys His Met
 10 20 30 40 50 60 70 80 90 100
 Leu Leu Trp Thr Ala Val Leu Asn Leu Ala Ala Gly Thr His Asp Leu Pro Lys Ala Val Val Lys Leu Glu Pro Pro Trp Ile Gln Val
 CTA CTG TGG ACA GCC GTG CTA AAT CTT GCT GCT GGG ACT CAT GAT CTT CCA AAG GCT GTG GTC AAA CTC GAG CCC CCG TGG ATC CAG GTG
 113 123 133 143 153 163 173 183 193
 Leu Lys Glu Asp Thr Val Thr Leu Thr Cys Glu Gly Thr His Asn Pro Gly Asn Ser Ser Thr Gln Trp Phe His Asn Gly Arg Ser Ile
 CTC AAG GAA GAC ACG GTG ACA CTG ACA TGC GAA GGG ACC ACC AAC CCT GGG AAC TCT TCT ACC CAG TGG TTT CAC AAT GGG AGG TCC ATC
 203 213 223 233 243 253 263 273 283
 Arg Ser Gln Val Gln Ala Ser Tyr Thr Phe Lys Ala Thr Val Asn Asp Ser Gly Glu Tyr Arg Cys Gln Met Glu Gln Thr Arg Leu Ser
 CGG AGC CAG GTC CAA GCC AGC TAC ACG TTT AAG GCC ACA GTC AAT GAC AGT GGA GAA TAT CCG TGT CAA ATG GAG CAG ACC CGC CTC AGC
 293 303 313 323 333 343 353 363 373
 Asp Pro Val Asp Leu Gly Val Ile Ser Asp Trp Leu Leu Gln Thr Pro Gln Leu Val Phe Leu Glu Gly Glu Thr Ile Thr Leu Arg
 GAC CCT GTA GAT CTG GGA GTG ATT TCT GAC TGG CTG CTC CAG ACC CCT CAG CTG GTG TTT CTG GAA GGG GAA ACC ATC ACG CTA Arg
 383 393 403 413 423 433 443 453 463
 Cys His Ser Trp Arg Asn Lys Leu Leu Asn Arg Ile Ser Phe Phe His Asn Glu Lys Ser Val Arg Tyr His His Tyr Ser Ser Asn Phe
 TGC CAT AGC TGG AGG AAC CTA CTG AAC AGG ATC TCG TTT TTT CAC AAT GAA AAA TCC GTG AGG TAT CAT CAC TAC AGT AGT AAT TTC
 473 483 493 503 513 523 533 543 553
 Ser Ile Pro Lys Ala Asn His Ser His Ser Gly Asp Tyr Tyr Cys Lys Lys Ser Leu Gly Arg Thr Leu His Gln Ser Lys Pro Val Thr
 TCT ATC CCC AAA GCC AAC CAC AGT CAC AGT GGG GAC TAC TAC TGC AAA GGA AGT CTA GGA AGC ACA CTG CAC CAG TCC AAG CCT GTC ACC
 563 573 583 593 603 613 623 633 643
 Ile Thr Val Gln Gly Pro Phe Thr Thr Thr Val Leu Arg Ala Ser Val Ser Pro Phe Pro Glu Gly Ser Leu Val Thr Leu Asn Cys
 ATC ACT GTC CAA GGG CCC TTC ACC ACG CCA GTG AGA GCA TCC GTG TCA TCT CCC TTC CCG GAG GGG AGT CTG GTC ACC CTG AAC TGT
 653 663 673 683 693 703 713 723 733
 Glu Thr Asn Leu Leu Gln Arg Pro Gly Leu Gln Leu His Phe Ser Phe Tyr Val Gly Ser Lys Ile Leu Glu Tyr Arg Asn Thr Ser
 GAG ACG AAT TTG CTC CTG CAG AGA CCC GGC TTA CAG CTT CAC TTC TCC TTC TAC GTG GGC AGC AAG ATC CTG GAG TAC AGG AAC ACA TCC
 743 753 763 773 783 793 803 813 823
 Ser Glu Tyr His Ile Ala Arg Ala Glu Arg Glu Asp Ala Gly Phe Tyr Trp Cys Glu Val Ala Thr Glu Asp Ser Val Leu Lys Arg
 TCA GAG TAC CAT ATA GCA AGG GCG GAA AGA GAA GAT GCT GGA TTC TAC TGG TGT GAG GTA GCC ACG GAG GAC AGC AGT GTC CTT AAG CGC
 833 843 853 863 873 883 893 903 913
 Ser Pro Glu Leu Glu Leu Val Leu Gly Pro Gln Ser Ser Ala Pro Val Trp Phe His Ile Leu Phe Tyr Leu Ser Val Gly Ile Met
 AGC CCT GAG TTG GAG CTC CAA GTG CTT GGT CCC CAG TCA TCA GCT CTT GTC TGG TTT CAC ATC CTG TTT TAT CTG TCA GTG GGA ATA ATG
 923 933 943 953 963 973 983 993 1003
 Phe Ser Leu Asn Thr Val Leu Tyr Val Lys Ile His Arg Leu Gln Arg Glu Lys Tyr Asn Leu Glu Val Pro Leu Val Ser Glu Gln
 TTT TCG TTG AAC ACG GTT CTC TAT GTG AAA ATA CAC AGG CTG CAG AGA GAG AAG AAA TAC AAC TTA GAA GTC CCT TTG GTT TCT GAG CAG
 1013 1023 1033 1043 1053 1063 1073 1083 1093
 Gly Lys Lys Ala Asn Ser Phe Gln Gln Val Arg Ser Asp Gly Val Tyr Glu Glu Val Thr Ala Thr Ala Ser Gln Thr Thr Pro Lys Glu
 GGA AAG AAA GCA AAT TCC TTT CAG CAA GTT AGA AGC GAT GGC GTG TAT GAA GAA GTA ACA GCC ACT CCG AGC CAG CAG ACA CCA AAA GAA
 1103 1113 1123 1133 1143 1153 1163 1173 1183
 Ala Pro Asp Gly Pro Arg Ser Val Gly Asp Cys Gly Pro Glu Gln Pro Glu Pro Leu Pro Pro Ser Asp Ser Thr Gly Ala Gln Thr
 GCG CCC GAT GGA CCT CGA AGC TCA GTG GGT GAC TGT GGA CCC GAG CAG CCT GAA CCC CTT CTT CTT CCG GAC AGT ACT GGG GCA CAA ACT
 1193 1203 1213 1223 1233 1243 1253 1263 1273
 Ser Gln Ser ***
 TCC CAA AGT TGA CCCTGAAACTGTGGGACCATGGCATGCACTCTTAATAAAGCAATATACAGACTGGAAAGGTCGACTCTAGAGATCCCCGCTCGAGAAJAA
 1283 1293 1303 1313 1323 1333 1343 1353 1363 1373

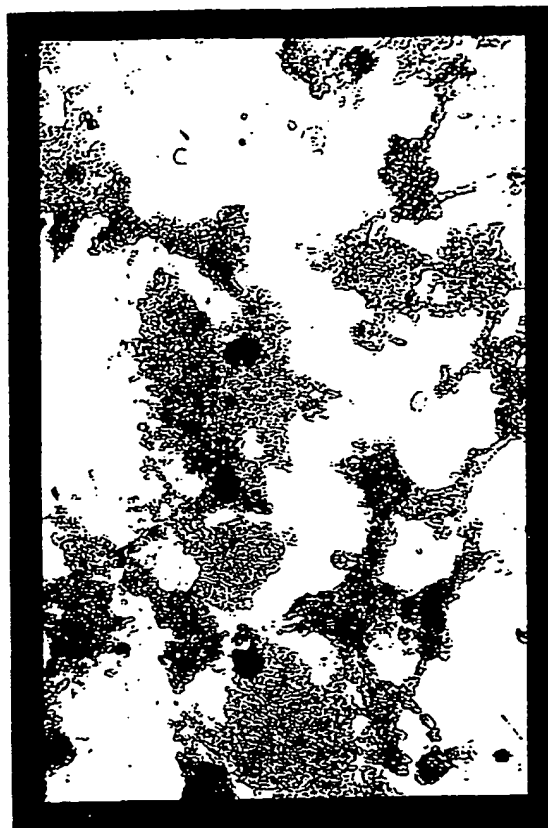
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FIGURE FOUR (A-D)

4a



4c



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4b



4d

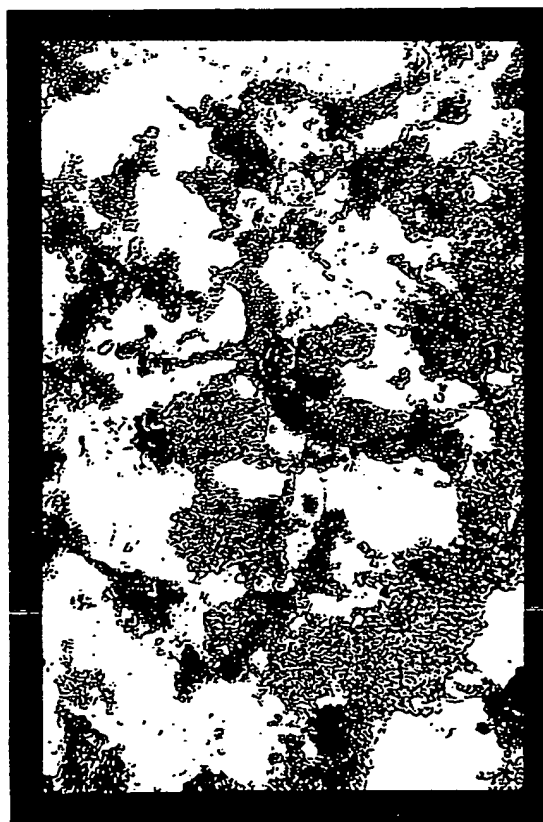
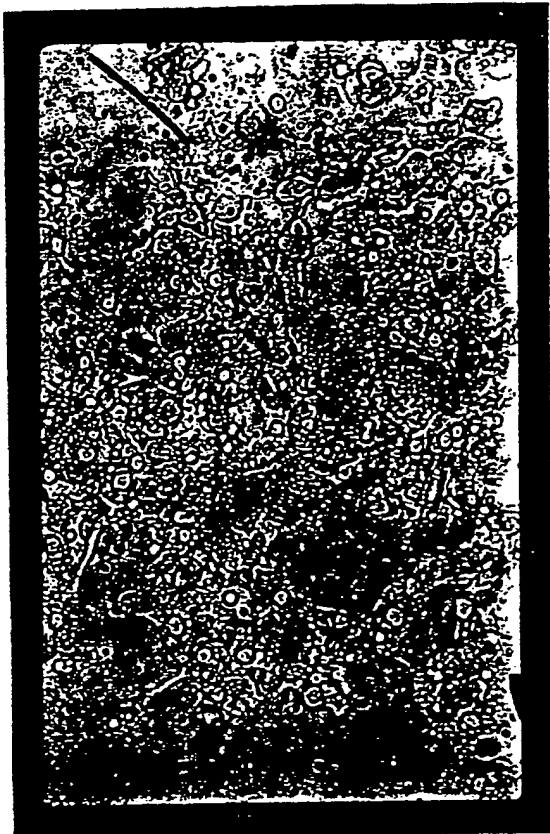


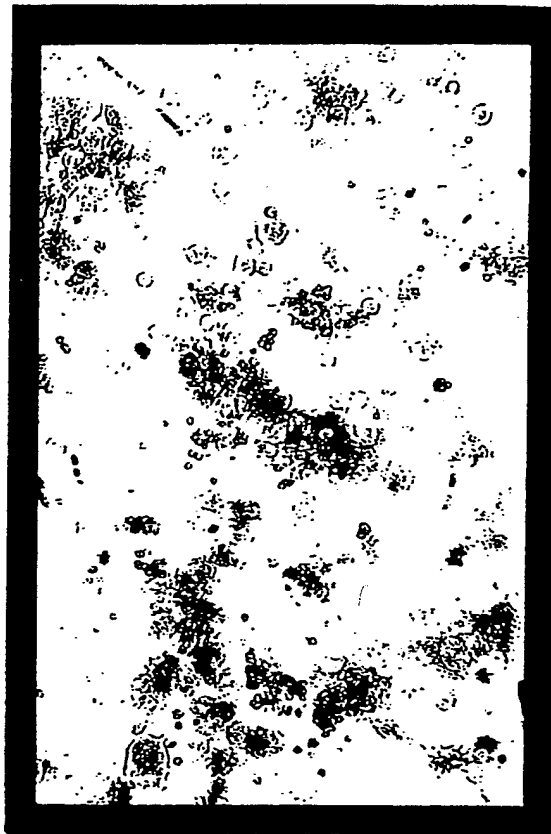
FIGURE FOUR (E-H)

4e



4g

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4f



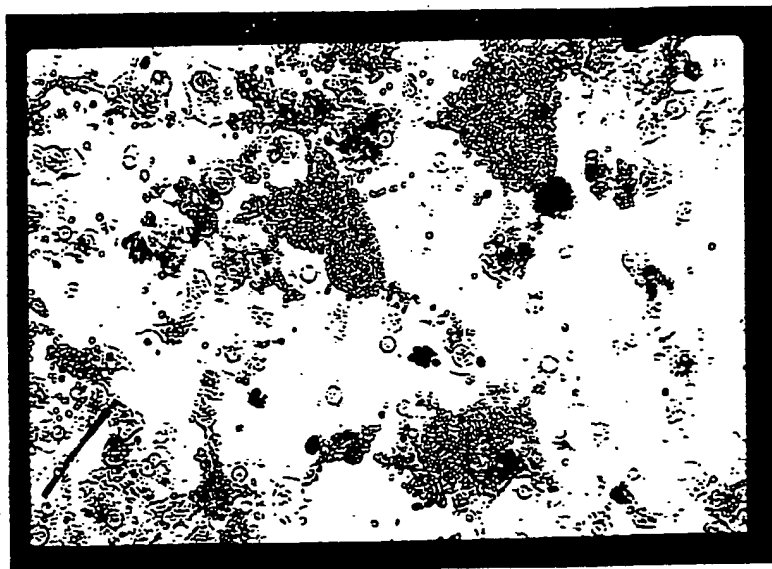
4g



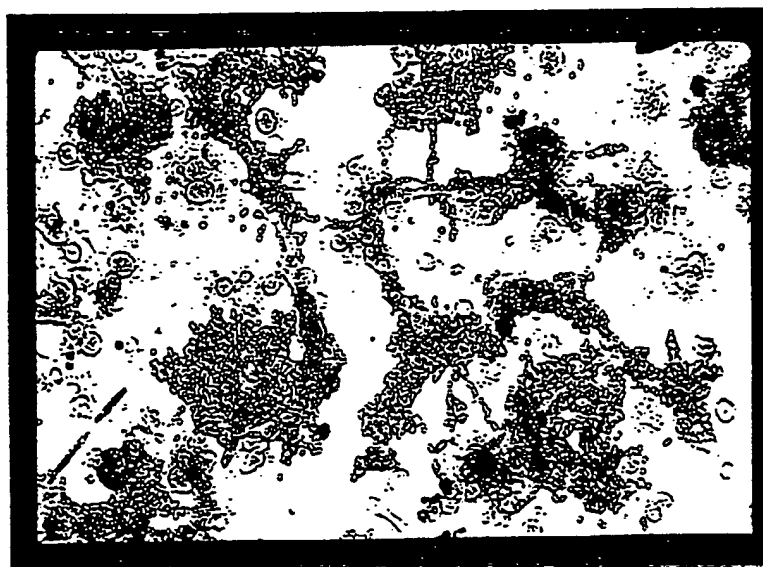
FIGURE FOUR (I-J)

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4i



4j



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FIGURE FIVE

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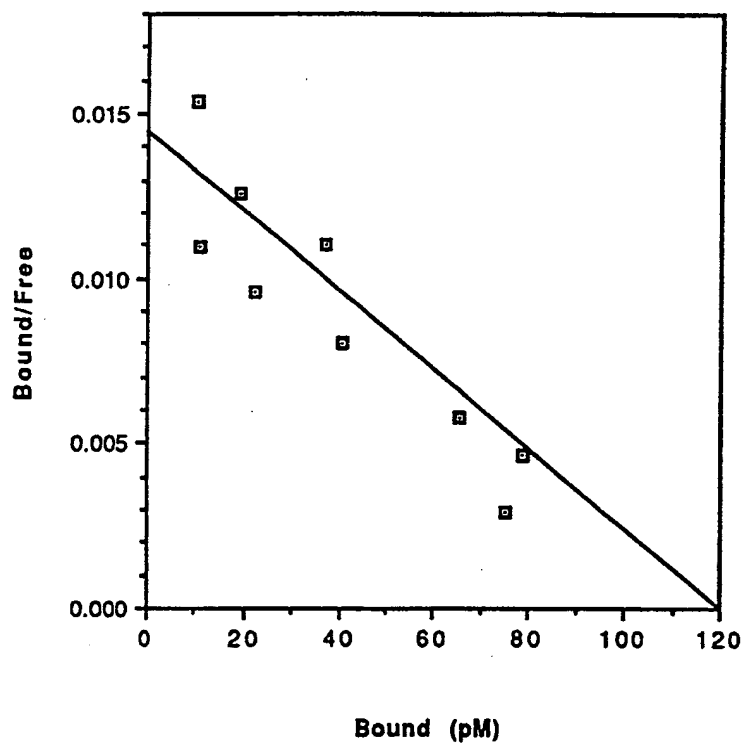


FIGURE SIX

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Met Glu Thr Gln Met Ser Gln Asn Val Cys Pro Arg Asn Leu Trp Leu Leu Gln Pro Leu Thr Val Leu Leu Leu Leu Ala	-10
GAATTCCTCACT ATG GAG ACC CAA ATG TCT CAG AAT GTA TGT CCC AGA AAC CTG TGG CTG CTT CAA CCA TTG ACA GTT TTG CTG GCT	92
NR 1	
Ser Ala Asp Ser Gln Ala Ala Pro Pro Lys Ala Val Leu Lys Leu Glu Pro Pro Trp Ile Asn Val Leu Gln Glu Asp Ser Val Thr	20
TCT GCA GAC AGT CAA GCT GCA GCT CCC CCA AAG GCT GTG CTG AAA CTT GAG CCC CCG TGG ATC AAC GTG CTC CAG GAG GAC TCT GTG ACT	182
Leu Thr Cys Gln Gly Ala Arg Ser Pro Glu Ser Asp Ser Ile Gln Trp Phe His Asn Gly Asn Leu Ile Pro Thr His Thr Gln Pro Ser	50
CTG ACA TGC CAG GGG GCT CGC AGC CCT GAG AGC GAC TCC ATT CAG TGG TTC CAC AAT GGG AAT CTC ATT CCC ACC CAC AGC CAG CCC AGC	272
Tyr Arg Phe Lys Ala Asn Asn Asp Ser Gly Glu Tyr Thr Cys Gln Thr Gly Gln Thr Ser Leu Ser Ser Asp Pro Val His Leu Thr Val	80
TAC AGG TTC AAG GCC AAC AAC AAT GAC AGC GGG GAG TAC ACG TGC CAG ACT GGC CAG ACC AGC CTC AGC GAC CCT GTG CAT CTG ACT GTG	362
Leu Ser Glu Trp Leu Val Leu Gln Thr Pro His Leu Glu Phe Gln Glu Gly Glu Thr Ile Met Leu Arg Cys His Ser Trp Lys Asp Lys	110
CTT TCC GAA TGG CTG CTC CHG ACC CCT CAC CTG CAG TTC CAG GAG GGA GAA ACC ATC ATG CTG AGG TGC CAC AGC TGG AAG GAC AAG	452
Pro Leu Val Lys Val Thr Phe Phe Gln Asn Gly Lys Ser Gln Lys Phe Ser Arg Leu Asp Pro Thr Phe Ser Ile Pro Gln Ala Asn His	140
CCT CTG GTC AAG GTC ACA TTC TTC CAG AAT GGA AAA TCC CAG AAA TTC TCC CGT TTG GAT CCC ACC TTC TCC ATC CCA CAA GCA AAC CAC	542
Ser His Ser Gly Asp Tyr His Cys Thr Gly Asn Ile Gly Tyr Thr Leu Phe Ser Ser Lys Pro Val Thr Ile Thr Val Gln Val Pro Ser	170
ACT CAC AGT GGT GAT TAC CAC TGC ACA GGA AAC ATA GGC TAC ACG CTG TTC TCA TCC AAG CCT GTG ACC ATC ACT GTC CAA GTG CCC AGC	632
Met Gly Ser Ser Pro Met Gly Ile Ile Val Ala Val Val Ile Ala Thr Ala Val Ala Ala Ile Val Ala Val Val Ala Leu Ile	200
ATG GGC AGC TCT TCA CCA ATG ATG GGC ATC ATT GTG GCT GTG GTC ATT GCG ACT GCT GTA GCA GCC ATT GTT GCT GCT GTA GTG GCC TTG ATC	722
Tyr Cys Arg Lys Lys Arg Ile Ser Ala Asn Ser Thr Asp Pro Val Lys Ala Ala Gln Phe Glu Pro Pro Gly Arg Gln Met Ile Ala Ile	230
TAC TGC AGG AAA AAG CGG ATT TCA GCC AAT TCC ACT GAT CCT GTG AAG GCT GCC CAA TT GAG CCA CCT GGA CGT CAA ATG ATT GCC ATC	812
Arg Lys Arg Gln Leu Glu Glu Thr Asn Asn Asp Tyr Glu Thr Ala Asp Gly Tyr Met Thr Leu Asn Pro Arg Ala Pro Thr Asp Asp	260
AGA AAG AGA CAA CTT GAA GAA ACC AAC AAT GAC TAT GAA ACA GCT GAC GGC GGC TAC ATG ACT CTG AAC CCC AGG GCA CCT ACT GAC GAT	902
Asp Lys Asn Ile Tyr Leu Thr Leu Pro Pro Asn Asp His Val Asn Ser Asn Asn ***	280
GAT AAA AAC ATC TAC CTG ACT CTT CCT CCC AAC GAC CAT GTC AAC AGT AAT AAC TAA AGAGTAAGCTTATGCCATGTGGTCATCTCTCAGCTACAGCTT	1002
GCTGATGGATGACAAANAGAGGGGAAATTTAAAGGAAATTTAAGGAGACTGGAAATCCTGAGCAAAACAAACCCACCTGGCCCTTAGAAATAGCTTACTTGGTTAACTACAA	1122
ACACAGCAAACTTACCGGCTCATCTACATACAGCATACAGCAAACTTACCTGGATTTCTGGTAAATGCTTAGTTAGAAATAGACAAACCCAGCAATCAAGACGCT	1242
ACTACATATATAGGTAGTACTAGGAGACTTCTAAGAGATACCTACCCCAAAACAAATATGTAATTTGAAACCCAGGATTCGCTTTATTTGCTTCCACATTTTCCCAATTAATA	1362
CTTGCGCTG	1370

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FIGURE SEVEN

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[illegible]

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FIGURE EIGHT (A-D)

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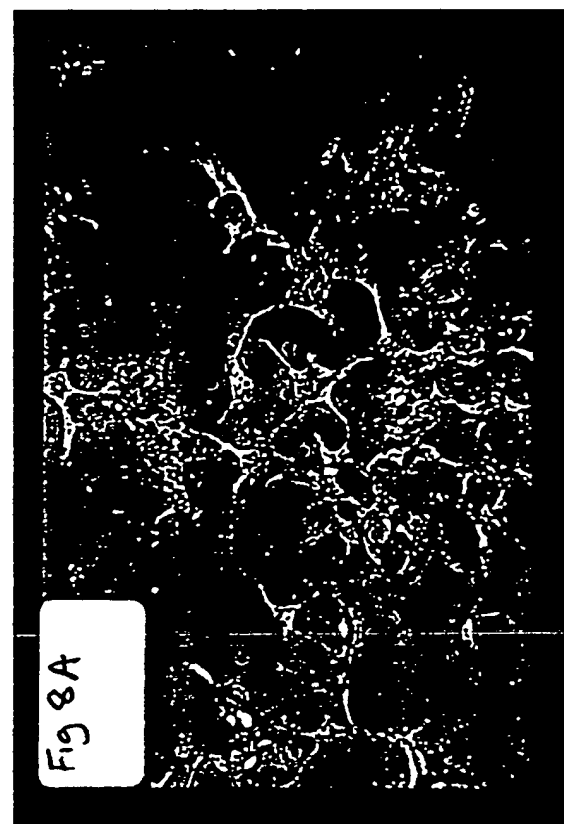
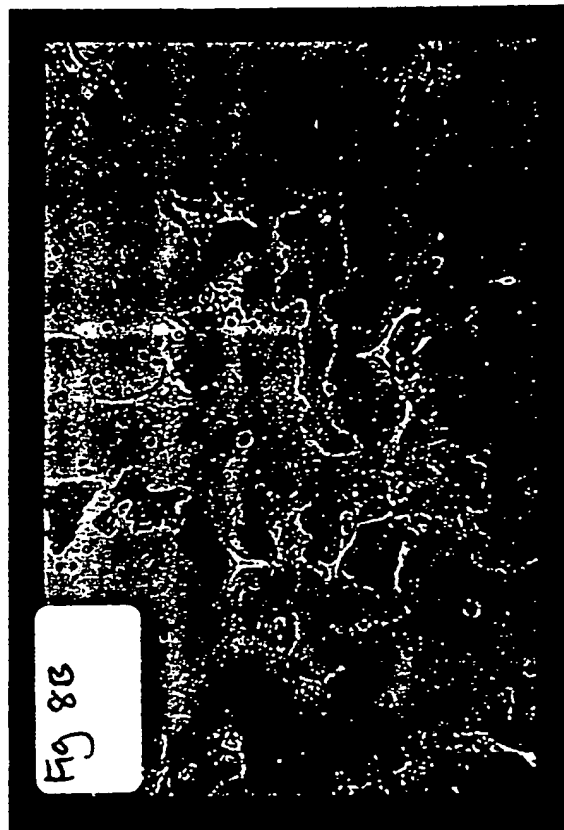


FIGURE EIGHT (E-H)

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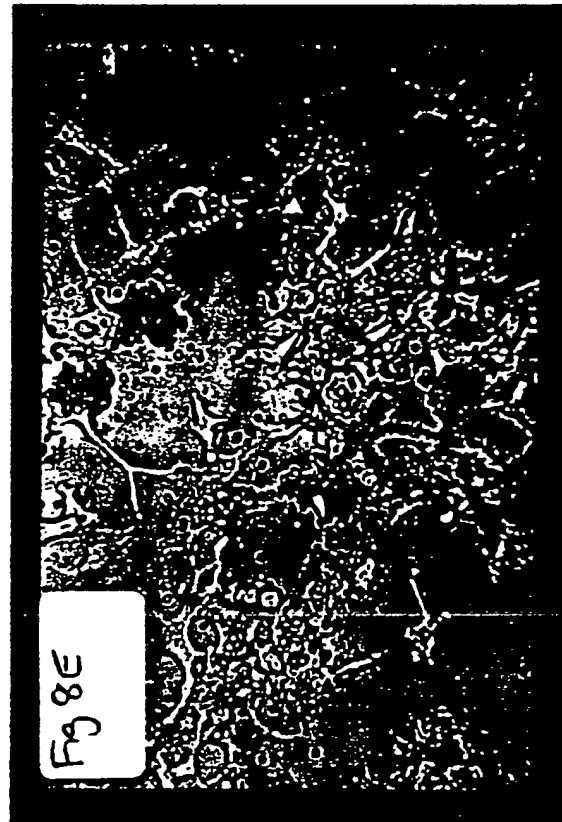
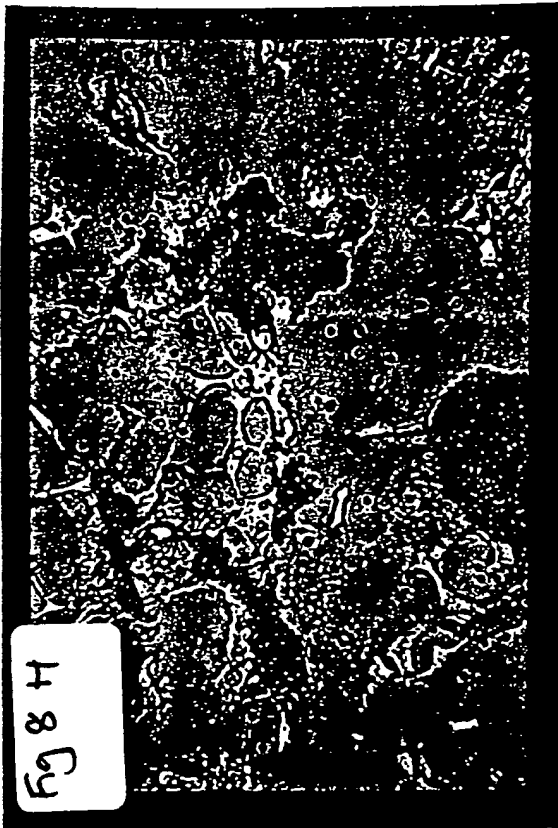
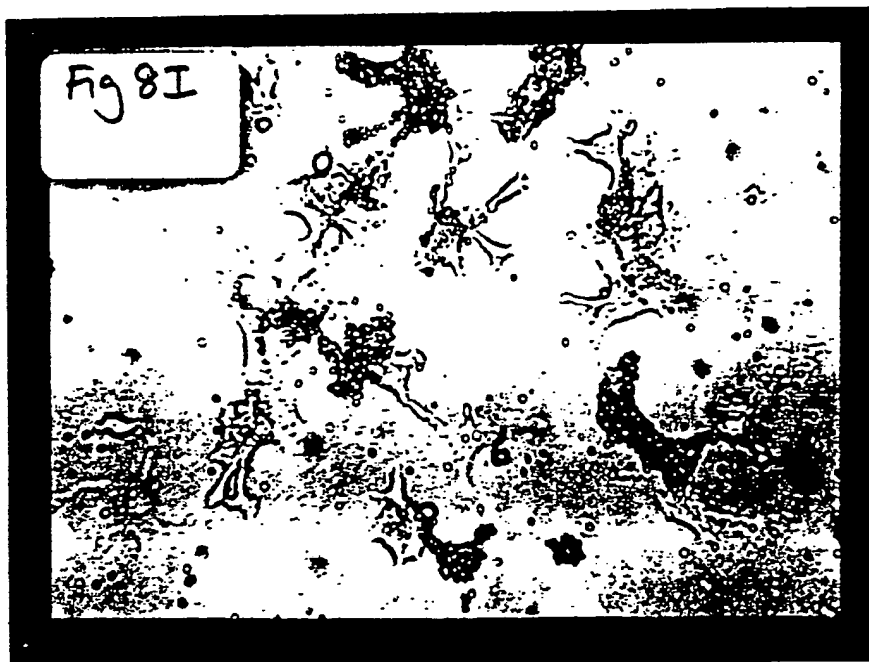


FIGURE EIGHT (I-J)

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FIGURE NINE

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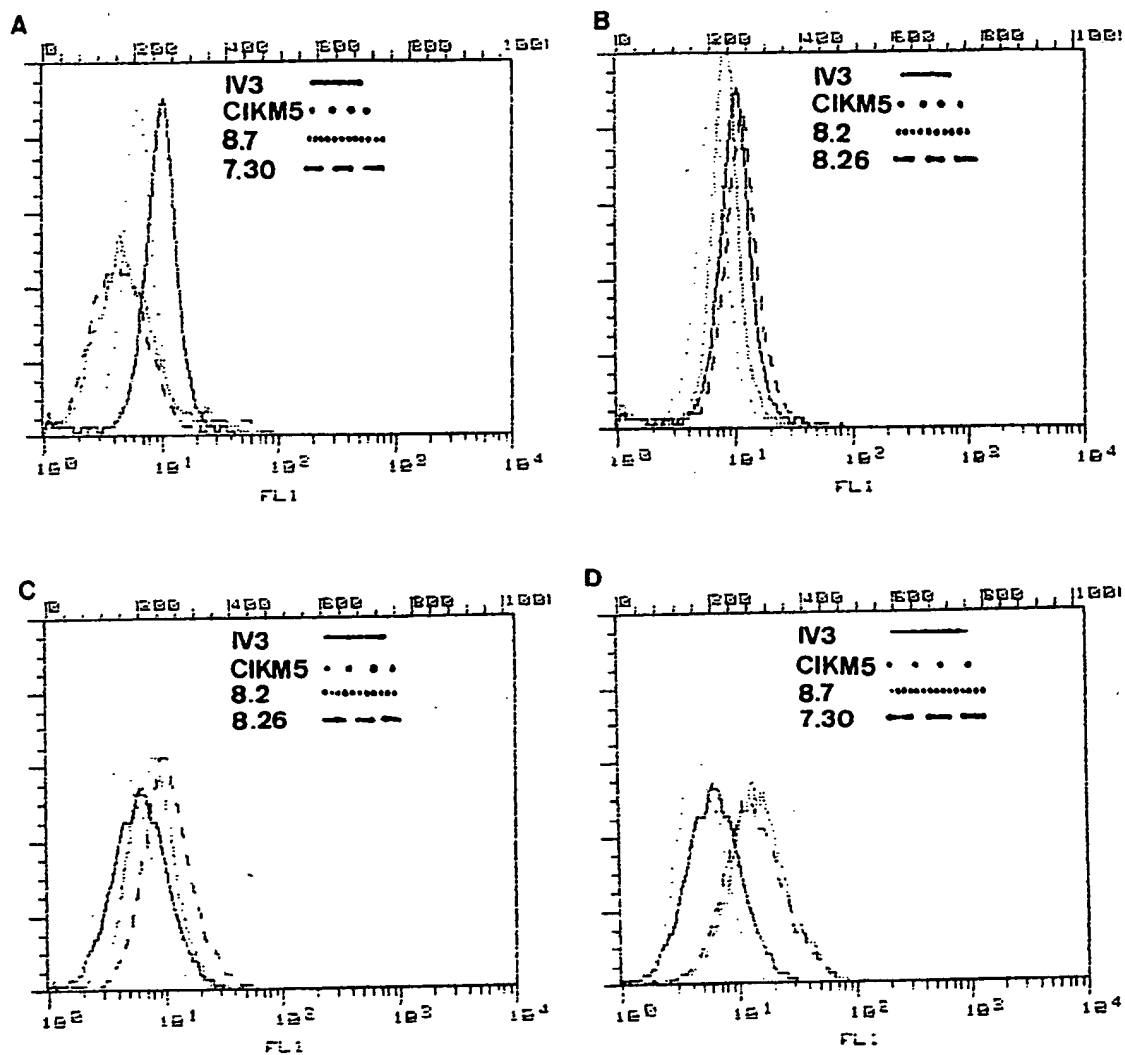


FIGURE TEN

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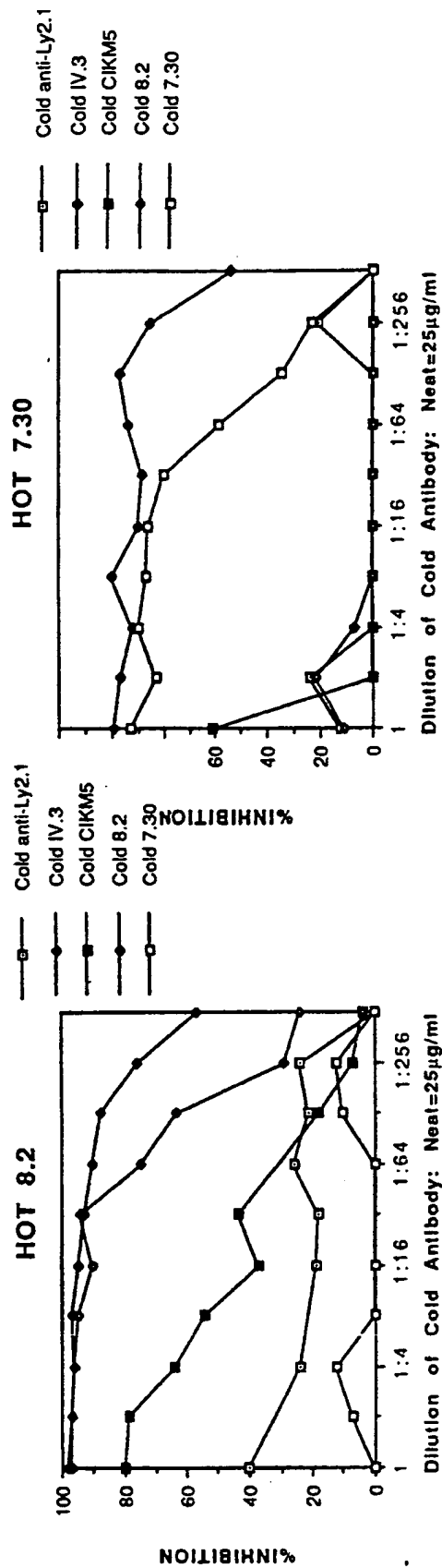
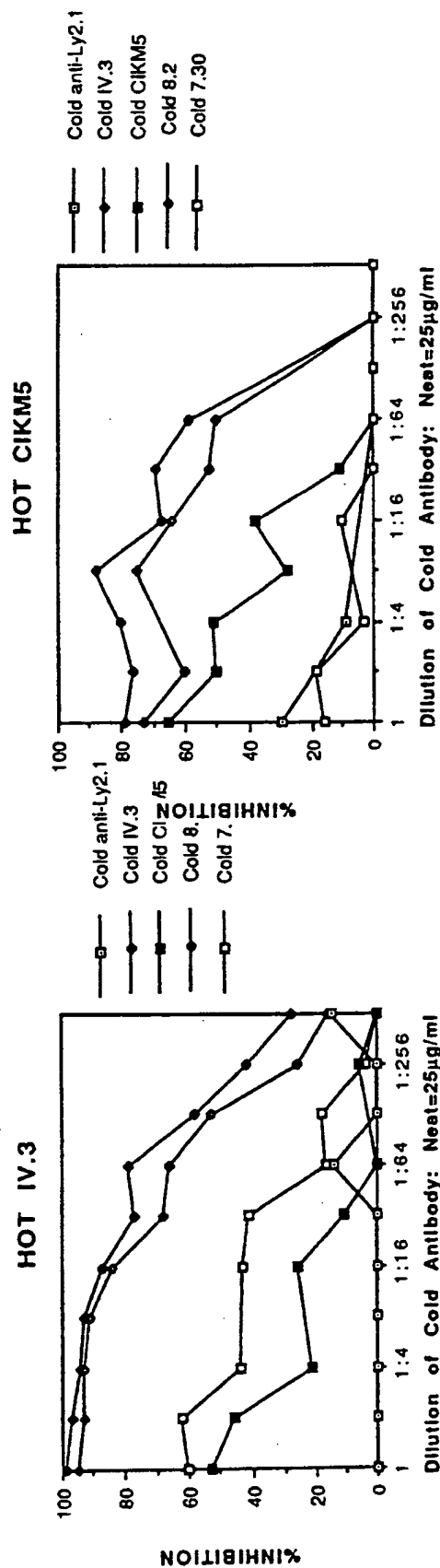
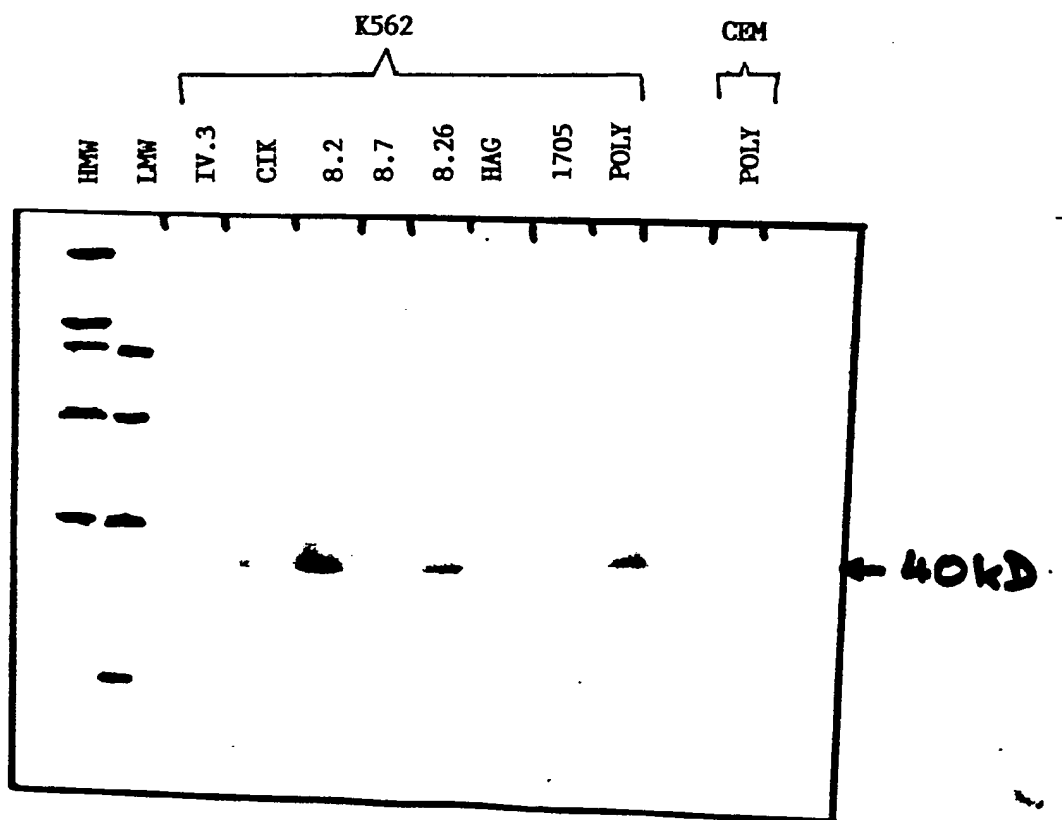


FIGURE ELEVEN A

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17.10.90 Immunpptn NP-40 O/N Exposure
10% reducing

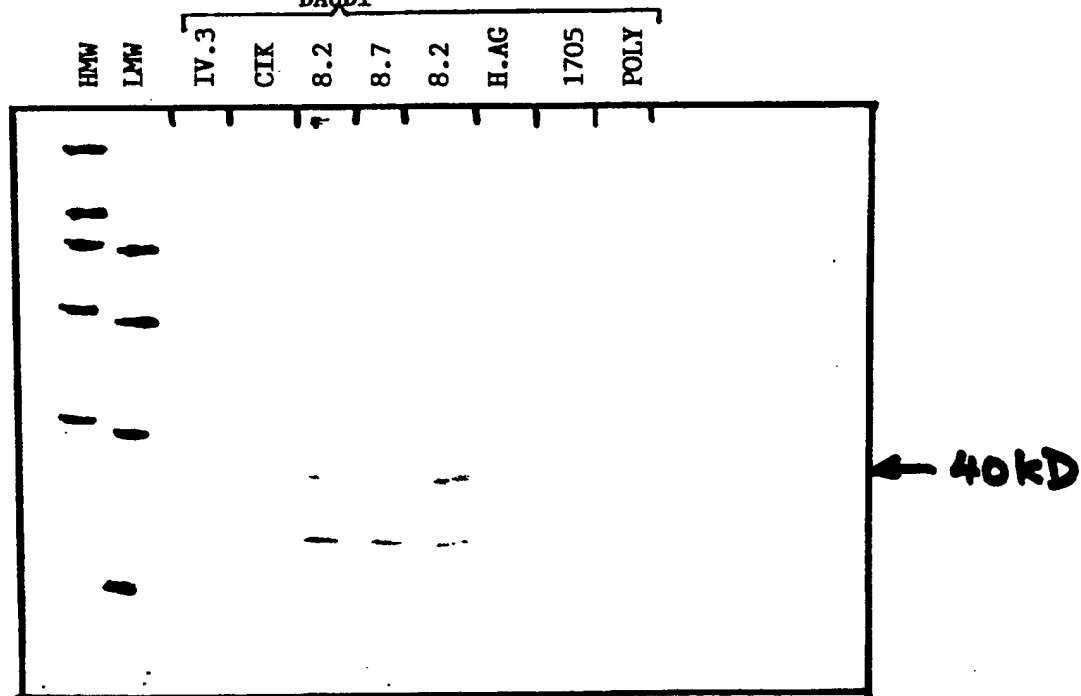


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FIGURE ELEVEN B

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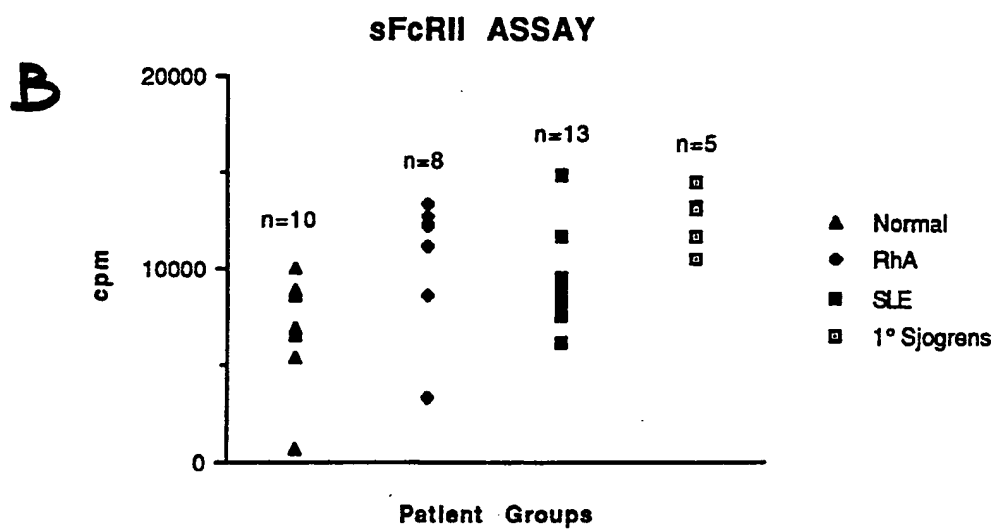
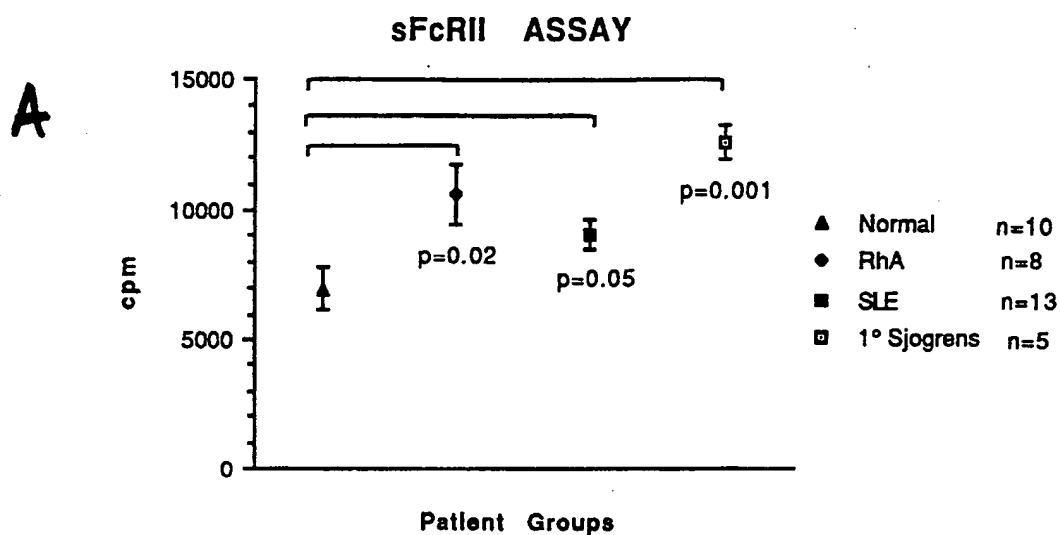
17.10.90 Immnpptn NP-40
10% reducing O/N Exposure
DAUDI



SUBSTITUTE SHEET

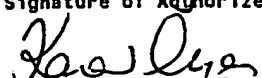
FIGURE TWELVE

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INTERNATIONAL SEARCH REPORT

International Application No. PCT/AU 90/00513

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) 6				
According to International Patent Classification (IPC) or to both National Classification and IPC				
Int. Cl. ⁵ C07K 15/06, 15/08, 15/12, C12N 15/13, C12P 21/08, G01N 33/563				
II. FIELDS SEARCHED				
Minimum Documentation Searched 7				
Classification System	Classification Symbols			
IPC	WPAT : Keywords : "Immunoglobulin", "Receptor", "Ig", "Fc", "FcR" USPA : Keywords : As above			
Documentation Searched other than Minimum Documentation to the extent that such Documents are Included in the Fields Searched 8				
AU: C07K 15/06, 15/08, 15/12, 13/00, C07G 7/00, C12N 15/12, 15/13, C12P 21/08 Chemical Abstracts : Keywords : "Immunoglobulin" or "Ig" and "Binding Molecule"; "Fc Receptor" or "FcR" and "Molecule"				
III. DOCUMENTS CONSIDERED TO BE RELEVANT 9				
Category*	Citation of Document, with indication, where appropriate, of the relevant passages 12	Relevant to Claim No 13		
A	AU,A, 32842/89 (SCHERING BIOTECH CORPORATION) 5 July 1989 (05.07.89)			
A	AU,A, 27830/88 (KISHIMOTO, T.) 13 July 1989 (13.07.89)			
A	AU,A, 82701/87 (MEMORIAL SLOAN-KETTERING CANCER INSTITUTE) 23 June 1988 (23.06.88)			
A	AU,A, 74881/87 (THE UNIVERSITY OF MELBOURNE) 4 February 1988 (04.02.88)			
A	AU,A, 63858/86 (MEDICAL BIOLOGY INSTITUTE) 14 April 1988 (14.04.88)			
A	EP,A, 269455 (TAKEDA CHEMICAL INDUSTRIES, LTD) 1 June 1988 (01.06.88)			
(continued)				
<p>* Special categories of cited documents: 10</p> <table style="width: 100%;"> <tr> <td style="width: 50%;"> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </td> <td style="width: 50%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&" document member of the same patent family</p> </td> </tr> </table>			<p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p>	<p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&" document member of the same patent family</p>
<p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p>	<p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&" document member of the same patent family</p>			
IV. CERTIFICATION				
Date of the Actual Completion of the International Search 4 February 1991 (04.02.91)		Date of Mailing of this International Search Report 11 February 1991		
International Searching Authority Australian Patent Office		Signature of Authorized Officer  K. AYERS		

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

A	Hibbs, M.L. et al. Proc. Natl. Acad. Sci. USA, Volume 85, issued April 1988, "Molecular cloning of a human immunoglobulin G Fc receptor" see pages 2240-2244
A	Shimizu, A. et al. Proc. Natl. Acad. Sci. USA, Volume 85, issued March 1988, "Human and rat mast cell high-affinity immunoglobulin E receptors: Characterization of putative α -chain gene products" see pages 1907-1911
P,A	Allen, J.M. and B. Seed. Science, Volume 243, issued 20 January 1989, "Isolation and expression of functional high-affinity Fc receptor complementary DNAs" see pages 378-381
A	Hibbs, M.L. et al. Proc. Natl. Acad. Sci. USA, Volume 83, issued September 1986, "The murine Fc receptor for immunoglobulin: Purification, partial amino acid sequence, and isolation of cDNA clones" see pages 6980-6984

V. [] OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE 1

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [] Claim numbers ..., because they relate to subject matter not required to be searched by this Authority, namely:
2. [] Claim numbers , because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. [] Claim numbers ..., because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4 (a):

VI. [] OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING 2

This International Searching Authority found multiple inventions in this international application as follows:

1. [] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.
2. [] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:
3. [] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:
4. [] As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

- [] The additional search fees were accompanied by applicant's protest.
 [] No protest accompanied the payment of additional search fees.

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON
INTERNATIONAL APPLICATION NO. PCT/AU 90/00513

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Members			
AU 32842/89	EP 319307	WO 8905351			
AU 27380/88	DK 7124/88 HU 50498 NO 885687 ZA 8809506	EP 321842 IL 88743 PT 89299	FI 885873 JP 2000443 EP 321601		
AU 82701/87	WO 8803172				
AU 74881/87	EP 268636	JP 63503386	WO 8707277		
EP 269455	JP 63246398				